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ORIGINAL ARTICLE

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Induction of histone acetylation and inhibition of growth by phenyl alkanoic acids and structurally related molecules

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Abstract *Purpose*: A structure-activity study was undertaken to determine the influence of side chain length of phenyl alkanoic acids and the degree of unsaturation of phenyl alkenoic acids on the induction of histone acetylation and inhibition of cancer cell proliferation. *Materials and methods*: Studies on cell proliferation were performed with DS19 mouse erythroleukemic cells, PC-3 human prostate cancer cells and Caco-2 human colon cancer cells. Actions on histone deacetylase and the induction of histone acetylation were compared for 4-phenylbutyrate and structurally related molecules. *Results*: Increasing inhibition of cell proliferation by phenyl alkanoic acids together with a decrease in cells in S phase and an increase in apoptotic cells was observed with increased chain length between four and ten carbons. Introduction of double bonds into the side chain was associated with increased growth inhibition. In contrast, 4-phenylbutyrate was a more potent inhibitor of histone deacetylase and inducer of histone acetylation than the other phenyl alkanoic acids examined. *Conclusions*: In comparison with the action of 4-phenylbutyrate, actions other than inhibition of histone deacetylase appear to be more important for growth inhibition by longer chain phenyl alkanoic and phenyl alkenoic acids.

Keywords Phenyl alkanoic acids · Histone deacetylase

Introduction

The identification of butyrate as an inhibitor of histone deacetylation [1, 24, 27] was followed after a number of years by the observation that 4-phenyl butyrate is also an inhibitor of histone deacetylase activity [15]. A challenge in this area of investigation is to describe compounds that will possess the growth-inhibitory and differentiation properties of butyrate but have rates of metabolism and pharmacokinetic properties that would permit use in cancer chemotherapy. 4-Phenylbutyrate is being investigated as a therapeutic agent for several types of cancer [7, 8, 9, 28]. We have previously observed that 4-phenylbutyrate is a more effective inhibitor of histone deacetylase and inducer of histone acetylation than several structural analogs including 2- and 3-phenylbutyrate, cinnamate, methoxycinnamate, 2-phenoxybutyrate and phenoxyacetate [16]. We extended these studies in the present work to examine the influence of side chain length on both histone acetylation and the inhibition of cell proliferation by phenyl alkanoic acids. The influence of side chain unsaturation and the substitution of a cyclohexyl group for a phenyl group were also studied. The results indicate that more potent inhibition of cell proliferation may be observed without a corresponding increase in the inhibition of histone deacetylase activity.

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Materials and methods

Cells and determination of cell proliferation

DS19 mouse erythroleukemia cells, PC-3 human prostate cancer cells and Caco-2 human colon cancer cells were incubated at 37°C in RPMI 1640 medium with 5% fetal calf serum and 25 m*M* *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) buffer. Morris hepatoma 7777 was transplanted bilaterally into a subcutaneous position in male Buffalo-strain rats. The induction, growth properties and histology of this tumor have been described [11, 21]. The rats were killed when the tumors had achieved a diameter of 1 to 2 cm. Experiments were performed in accordance with "Principles of laboratory animal care" (NIH publication no. 85-23, revised 1985).

Proliferation of DS19 cells was measured by plating the cells at an initial density of 10^5 per milliliter and counting cell density at 24-h intervals for 72 h using a hemocytometer. The incorporation of [^{3}H]thymidine into DNA was measured after incubating the cells

for 2 h with 2 μCi [^{3}H]thymidine. Cells were washed with 0.9% NaCl and with 10% trichloroacetic acid and 5% sodium pyrophosphate. Cells were detached and RNA was hydrolyzed by incubating for 1 h at 37°C with 0.33 *N* KOH. DNA was precipitated by addition of an equal volume of 20% trichloroacetic acid and 10% sodium pyrophosphate. The precipitate was washed with 10% trichloroacetic acid and 5% sodium pyrophosphate, and DNA was hydrolyzed by incubation with 5% trichloroacetic acid at 90°C for 20 min. Radioactivity of the acid-soluble material was measured by liquid scintillation counting.

Cell cycle parameters

Cells were fixed with 70% ethanol and were then treated with ribonuclease and stained with propidium iodide. Fluorescence activated cell sorting was performed with a Becton Dickinson FACSCalibur instrument and the data were analyzed using Cell Quest 3.2 software. Subdiploid cells were recorded as apoptotic cells.

Reagents

4-Phenylbutyric acid was purchased from Aldrich Chemical Company, Milwaukee, Wis. Other phenyl alkanoic acids and phenyl alkenoic acids were provided by CircaGen Pharmaceutical, Phoenix, Md. These compounds were studied as their sodium salts.

Histone deacetylase assay

Activity of histone deacetylase was measured by the liberation of [^{3}H]acetate from labeled acetylated histones as previously described [15]. Cells were centrifuged and washed once with phosphate-buffered saline. The pelleted cells were homogenized in 10 m*M* Tris-HCl, pH 7.8, 3 m*M* magnesium chloride and 20 m*M* sodium chloride. A crude nuclear fraction was obtained by centrifugation of the homogenate for 5 min at 625 *g* and resuspending the pellet in 10 m*M* Tris-HCl, pH 7.8, 3 m*M* magnesium chloride and 20 m*M* sodium chloride. Incubations at 37°C were performed in a total volume of 100 μl containing 5 μg ^{3}H-labeled histones (3000 cpm/μg) in 10 m*M* Tris-HCl, pH 7.8, 3 m*M* magnesium chloride, 20 m*M* sodium chloride and nuclear fraction derived from homogenate containing 50 μg protein. Incubations for 0–30 min were stopped by the addition of 10 μl concentrated hydrochloric acid. Then 1 ml ethyl acetate was added. The mixture was vortexed and centrifuged for 2 min, and 0.8 ml of the top layer was taken for liquid scintillation counting.

Histone isolation and electrophoresis

The isolation of histones and electrophoresis on urea-acetic acid polyacrylamide gels was performed as previously described [14]. The relative levels of acetylated H4 histones were quantitated by densitometry of Coomassie-blue-stained gels. The percentages of H4 histone with 0, 1, 2, 3 or 4 acetyl groups are presented in the Results section as H4-0, H4-1, H4-2, H4-3 and H4-4, respectively.

The procedure for immunoblotting of polyacrylamide gels was performed as described previously [16]. Rabbit polyclonal antibody against acetylated H2A (catalog number AHP419) was obtained from Serotec, Raleigh, N.C.

Statistical evaluation

The significance of differences in the results was determined by a two-tailed Student's *t*-test or by Dunnett's test using the Instat program. Probabilities less than 5% were considered significant.

Results

The data shown in Table 1 suggest that increasing the chain length of phenyl alkanoic acids from four carbons in 4-phenylbutyrate to ten carbons in 10-phenyloctanoate resulted in a greater inhibition of the proliferation of DS19 mouse erythroleukemia cells. On the other hand, there was a decrease in the inhibition of histone deacetylase activity with longer side chains. In the case of 4-phenylbutyrate, the IC_{50} for inhibition of histone deacetylase in isolated nuclei was less than the IC_{50} for inhibition of cell proliferation. For the longer chain phenyl alkanoic acids examined the IC_{50} for inhibition of cell proliferation was less than the IC_{50} for inhibition of histone deacetylase. Comparison of the IC_{50} for inhibition of cell proliferation suggested that the presence of unsaturated bonds in the alkanoic chain can result in greater inhibition as judged by the relative values for 5-phenylpentanoate and 5-phenylpenta-2,4-dienoate and for 7-phenylheptanoate and 7-phenylhepta-2,4,6-trienoate.

The data shown in Table 2 indicate that with increased side chain length from four to ten carbons, the phenyl alkanoic acids caused a greater decrease in the percentage of diploid DS19 cells in S phase and an increase in the percentage of cells in the G_1 phase of the cell cycle. At concentrations of 0.5 and 1.0 m*M* there was a large increase in the percentage of apoptotic cells with side chains of eight and ten carbons. An increase in the percentage of apoptotic cells was also seen with 1 m*M* 7-phenylhepta-2,4,6-trienoate (CG1520) relative to that seen with a saturated side chain.

A greater growth-inhibitory effect by longer chain phenyl alkanoic acids occurred in Caco-2 colon cancer cells and PC-3 prostate cancer cells (Table 3). Compounds with eight and ten carbons caused a much greater inhibition of [^{3}H]thymidine incorporation into DNA when incubated for 48 h at a concentration of 1 m*M* than phenyl alkanoic acids having chain lengths of five, six or seven carbons.

An examination of the induction of histone H4 acetylation in DS19 cells after a 2-h incubation with

Table 1 Inhibition of growth of DS19 cells and of histone deacetylase (HDAC) activity by phenyl alkanoic acids and phenyl alkenoic acids

Compound	IC_{50} (m*M*)	
	Growth	HDAC
4-Phenylbutyrate	1.31	0.62
5-Phenylpentanoate	0.97	1.4
6-Phenylhexanoate	1.14	> 2.0
7-Phenylheptanoate	0.70	1.3
8-Phenyloctanoate	0.28	1.95
10-Phenyldecanoate	0.26	1.7
4-Cyclohexylbutyrate	1.1	> 1.0
5-Phenylpenta-2,4-dienoate (CG-1255)	0.13	0.96
7-Phenylhepta-2,4,6-trienoate (CG-1520)	0.35	> 1.0

Table 2 Effect of phenyl alkanoic and alkenoic acids on cell cycle parameters. DS19 cells (1.0×10^6 cells in 2.5 ml medium) were incubated for 24 h. The values presented are the means ± SD for the number of flasks

Phenyl alkanoic or alkenoic acid	No. of flasks	Concentration (m*M*)	% diploid cells in phases of the cell cycle			% apoptosis
			G_1	S	G_2/M	
Control	20		28.0 ± 6.8	62.6 ± 7.5	9.4 ± 3.0	1.0 ± 1.5
Phenylbutyrate	6	0.5	29.6 ± 5.5	60.4 ± 5.5	10.0 ± 2.7	1.9 ± 1.3
	4	1.0	33.3 ± 1.0	60.6 ± 0.7	6.2 ± 1.1	0.3 ± 0.5
Phenylpentanoate	4	0.5	34.9 ± 1.6	58.8 ± 3.3	6.3 ± 1.6	1.1 ± 0.5
	4	1.0	41.5 ± 1.1**	44.1 ± 3.4**	14.4 ± 3.7	0 ± 0
Phenylhexanoate	6	0.5	37.9 ± 1.4*	54.3 ± 3.0	7.9 ± 3.2	1.7 ± 2.2
	4	1.0	41.2 ± 5.7**	46.7 ± 3.6**	12.1 ± 2.3	0.5 ± 0.6
Phenylheptanoate	6	0.2	29.6 ± 3.8	60.8 ± .4.5	9.7 ± 1.5	2.5 ± 3.2
	4	0.5	48.5 ± 3.2**	41.6 ± 0.4**	9.9 ± 3.3	4.3 ± 2.7
	4	1.0	36.8 ± 1.6	49.3 ± 2.1**	13.9 ± 1.5	0.8 ± 0.9
Phenyloctanoate	4	0.2	38.3 ± 0.7*	54.0 ± 0.7	7.7 ± 0.4	0 ± 0
	4	0.5	34.7 ± 1.8	63.6 ± 1.1	1.7 ± 1.3**	13.9 ± 1.8**
	4	1.0	54.5 ± 6.5**	38.9 ± 4.2**	6.6 ± 7.6	46.0 ± 23.9**
Phenyldecanoate	4	0.2	34.5 ± 3.1	59.3 ± 1.8	6.2 ± 1.6	7.7 ± 1.3
	4	0.5	60.0 ± 11.7**	40.1 ± 11.7**	0 ± 0**	52.0 ± 17.0**
	4	1.0	65.7 ± 2.8**	33.4 ± 4.0**	0.8 ± 1.7**	40.9 ± 10.2**
CG1255	6	0.5	39.3 ± 10.9**	49.2 ± 6.1**	11.4 ± 8.4	2.0 ± 1.9
	4	1.0	23.2 ± 3.9	74.1 ± 2.2*	2.7 ± 2.9*	2.8 ± 3.3
CG1520	4	0.5	41.5 ± 2.4**	49.9 ± 8.0**	8.6 ± 6.2	2.8 ± 0.2
	4	1.0	69.2 ± 13.0**	29.3 ± 13.2**	1.5 ± 0.5**	19.7 ± 0.9**

*$P < 0.05$, **$P < 0.01$, relative to control values as determined by Dunnett's test

Table 3 Effect of phenyl alkanoic acids on the incorporation of [^{3}H]thymidine into DNA in Caco-2 human cancer cells and PC-3 human prostate cancer cells. Cells (0.5×10^6) were plated and the medium was changed after 24 h. The cells were incubated with phenyl alkanoic acids in a volume of 5 ml for 48 h before addition of 5 μCi [^{3}H]thymidine. Incubations were stopped after a further 2 h. The values presented are the means ± SD for three flasks

Cells	Phenyl alkanoic acid (1 m*M*)	cpm per flask	Percent of control
Caco-2	Control	27,114 ± 3,126	100
	Phenylpentanoate	17,983 ± 3,475*	66
	Phenylhexanoate	14,796 ± 644**	55
	Phenylheptanoate	8,455 ± 1,401**	31
	Phenyloctanoate	384 ± 21***	1
	Phenyldecanoate	27 ± 3***	0
PC-3	Control	41,245 ± 4,226	100
	Phenylpentanoate	37,085 ± 804	90
	Phenylhexanoate	15,237 ± 1,121***	37
	Phenylheptanoate	9,754 ± 1,974***	24
	Phenyloctanoate	1,649 ± 515***	4
	Phenyldecanoate	11 ± 3***	0

*$P < 0.05$, **$P < 0.005$, ***$P < 0.0005$, relative to control values

1 m*M* phenyl alkanoic acids revealed that the effect of phenylbutyrate was greater than the action of phenyl alkanoic acids with a chain length between five and ten carbons (Table 4). Similarly, there was increased acetylation of H4 histone when PC-3 prostate cancer cells were incubated with 1 m*M* 4-phenylbutyrate and a smaller effect with 5-phenylpentanoate, but no significant effect with longer chain phenyl alkanoic acids (data not shown). In accordance with our previous observation [17], the acetylation of H4 histone was increased in control DS19 cells when the incubation was continued for 24 h at high cell density (Table 5). Under these conditions there was still an enhancing effect on acetylation with 1 m*M* phenylbutyrate that was greater than that seen with chain lengths of five, six or seven carbons. With chain lengths of eight or ten carbons and prolonged incubation, toxicity was manifest in cell morphology and there was decreased histone acetylation. By decreasing the initial cell density it was possible to avoid the increase in histone acetylation in control cells (Table 6). It may be noted that these cells had approximately a 12-h doubling time in logarithmic growth. With a low initial cell density the levels of histone acetylation in control cells and cells treated with phenyl alkanoic acids were similar after 2-h or 24-h incubations (Tables 4 and 6) and confirmed the greater induction of histone acetylation seen with phenylbutyrate than with longer chain phenyl alkanoic acids. A greater effect on histone acetylation with phenylbutyrate than with longer chain phenyl alkanoic acids was also observed in studies with PC-3 human prostate cancer cells (data not shown).

The data shown in Tables 4 and 7 indicate that a change in the ring portion of the molecule decreased the induction of histone acetylation relative to that induced by 4-phenylbutyrate. Changing the ring from a phenyl to a cyclohexyl group resulted in a compound with a very limited effect on histone H4 acetylation when incubated at concentrations of 0.1, 0.3 and 1.0 m*M*. A greater effect on histone H4 acetylation was seen with 5-phenylpenta-2,4-dienoate when incubated at the same concentrations. There was no significant effect of 7-phenylhepta-2,4,6-trienoate on H4 histone acetylation in DS19 cells when examined after a 2-h incubation at concentrations of 0.1, 0.5 or 1.0 m*M* (data not shown). A statistically significant decrease in unacetylated H4

Table 4 H4 histone acetylation in DS19 mouse erythroleukemia cells incubated with phenylalkanoic acids at a concentration of 1 m*M*. DS19 cells (20×10^6) were incubated for 2 h in 20 ml medium before histone isolation and electrophoresis. The values shown are the percentages of unacetylated and acetylated H4 histones in relation to total H4 histone, and are the means ± SD for the number of determinations given in parentheses

Alkanoate chain length	Percent of total H4 histone				
	H4-0	H4-1	H4-2	H4-3	H4-4
Control ($n=9$)	68.9 ± 6.6	23.9 ± 5.4	3.3 ± 2.1	0.1 ± 0.1	3.8 ± 0.5
4C ($n=4$)	45.8 ± 2.3***	36.8 ± 2.9***	11.6 ± 3.0***	2.7 ± 0.6**	3.1 ± 0.9
5C ($n=3$)	57.9 ± 4.4*	29.8 ± 0.9	7.7 ± 3.3*	0.6 ± 0.7*	3.5 ± 0.4
6C ($n=3$)	59.9 ± 2.2*	33.3 ± 0.8*	2.8 ± 1.6	0.5 ± 0.5	3.5 ± 0.7
7C ($n=6$)	57.8 ± 6.9*	32.5 ± 4.9*	5.8 ± 3.5	0.5 ± 0.5*	3.4 ± 0.5
8C ($n=4$)	53.6 ± 1.3**	36.7 ± 1.3**	6.9 ± 1.0*	0.2 ± 0.3	2.8 ± 0.4**
10C ($n=3$)	61.7 ± 6.3	31.0 ± 3.8	4.6 ± 2.7	0.3 ± 0.5	2.5 ± 1.6

*$P<0.05$, **$P<0.005$, ***$P<0.0005$, relative to control values

Table 5 H4 histone acetylation in DS19 mouse erythroleukemia cells incubated at high density with phenylalkanoic acids at a concentration of 1 m*M*. DS19 cells (20×10^6) were incubated for 24 h in 20 ml medium before histone isolation and electrophoresis. The values shown are the percentages of unacetylated and acetylated H4 histones in relation to total H4 histone, and are the means ± SD for the number of determinations given in parentheses

Alkanoate chain length	Percent of total H4 histone				
	H4-0	H4-1	H4-2	H4-3	H4-4
Control ($n=11$)	48.3 ± 2.6	32.5 ± 3.1	12.3 ± 2.7	2.5 ± 1.5	4.3 ± 0.7
4C ($n=4$)	29.4 ± 1.2***	36.8 ± 1.8*	22.0 ± 1.3***	7.7 ± 0.8***	4.2 ± 0.5
5C ($n=6$)	40.3 ± 3.9***	34.9 ± 1.5	16.5 ± 2.9*	4.2 ± 1.7	4.3 ± 0.8
6C ($n=3$)	40.3 ± 1.0***	37.8 ± 1.9*	15.8 ± 0.5	2.7 ± 0.9	3.5 ± 1.2
7C ($n=5$)	44.4 ± 2.1*	38.2 ± 2.7**	11.5 ± 2.4	2.2 ± 1.2	3.7 ± 0.8
8C ($n=4$)	69.1 ± 1.4***	28.6 ± 1.7*	1.0 ± 0.7***	0.8 ± 0.5*	0.5 ± 0.3***
10C	Cell death, histones appeared degraded				

*$P<0.05$, **$P<0.005$, ***$P<0.0005$, relative to control values

Table 6 H4 histone acetylation in DS19 mouse erythroleukemia cells incubated at low density with phenylalkanoic acids at a concentration of 1 m*M*. DS19 cells (5×10^6) were incubated for 24 h in 20 ml medium before histone isolation and electrophoresis. The values shown are the percentages of unacetylated and acetylated H4 histones in relation to total H4 histone, and are the means ± SD for the number of determinations given in parentheses

Alkanoate chain length	Percent of total H4 histone				
	H4-0	H4-1	H4-2	H4-3	H4-4
Control ($n=9$)	67.6 ± 5.1	25.8 ± 4.0	3.5 ± 2.0	0.2 ± 0.1	2.9 ± 0.5
4C ($n=3$)	43.5 ± 6.1***	36.4 ± 3.4**	12.7 ± 7.0**	3.8 ± 2.3**	3.6 ± 0.2*
5C ($n=3$)	59.2 ± 2.2*	30.4 ± 4.7	4.3 ± 0.5	0.9 ± 0.6*	5.1 ± 3.1
6C ($n=3$)	64.8 ± 2.6	30.5 ± 1.0	1.1 ± 1.0	1.5 ± 1.2	2.0 ± 1.0
7C ($n=3$)	62.3 ± 1.8	31.9 ± 1.0*	1.9 ± 0.6	1.1 ± 1.2	2.9 ± 1.1

*$P<0.05$, **$P<0.005$, ***$P<0.0005$, relative to control values

histone was seen in rat liver and Morris hepatoma 7777 when tumor-bearing rats received injections of 5-phenylpenta-2,4-dienoate (Table 8). Under the conditions studied, the decrease in unacetylated H4 histone was not statistically significant in spleen and kidney of the tumor-bearing rats.

The reversibility of the action of 5-phenylpenta-2,4-dienoate was addressed by the study recorded in Table 9. The data confirmed the induction of H4 histone acetylation caused by incubation of DS19 cells with 1 m*M* 5-phenylpenta-2,4-dienoate for 2 h and indicate that this effect was essentially reversed when the cells were resuspended in fresh medium and incubated for a further 6 h. The induction of increased acetylation of core nucleosomal histones by 5-phenylpenta-2,4-dienoate was not restricted to the H4 histones. This is illustrated in Fig. 1 which is an immunoblot showing increased acetylation of the ubiquitinated H2A histone after incubation of DS19 cells with 0.1, 0.3 and 1.0 m*M* 5-phenylpenta-2,4-dienoate for 24 h.

Table 7 H4 histone acetylation in DS19 mouse erythroleukemia cells incubated with 4-cyclohexylbutyrate (CG1121) or 5-phenylpenta-2,4-dienoate (CG1255). DS19 cells (15×10^6) were incubated for 2 h in 20 ml medium before histone isolation and electrophoresis. The values shown are the percentages of unacetylated and acetylated H4 histones in relation to total H4 histone, and are the means ± SD for the number of determinations

	Concentration (m*M*)	No. of determinations	Percent of total H4 histone				
			H4-0	H4-1	H4-2	H4-3	H4-4
Control		19	72.4 ± 5.8	20.9 ± 6.8	1.8 ± 1.3	0.8 ± 1.2	4.1 ± 2.1
CG1121	0.1	3	65.4 ± 6.9	25.3 ± 5.9	3.9 ± 1.8*	0.8 ± 0.3	4.7 ± 1.0
	0.3	5	67.2 ± 7.5	24.4 ± 6.1	4.3 ± 1.9**	0.9 ± 0.6	3.2 ± 2.1
	1.0	5	70.9 ± 9.5	17.8 ± 11.3	6.6 ± 3.8**	1.1 ± 0.9	3.6 ± 2.0
CG1255	0.1	5	62.4 ± 2.7**	28.5 ± 1.4*	5.3 ± 2.9**	0.4 ± 0.2	3.3 ± 1.0
	0.3	4	54.3 ± 1.9**	33.9 ± 2.1**	8.3 ± 1.3**	0.6 ± 0.7	3.0 ± 1.6
	1.0	4	52.0 ± 4.8**	32.3 ± 5.1*	10.3 ± 1.9**	2.0 ± 1.0	3.5 ± 0.8

*$P < 0.05$, **$P < 0.005$, relative to control values

Table 8 Acetylation of H4 histone in tissues of rats treated with 5-phenylpenta-2,4-dienoate (CG1255). The data presented are the percentages of H4 histones with 0 to 4 acetyl groups in control and treated rats bearing subcutaneous transplants of Morris hepatoma 7777. 5-Phenylpenta-2,4-dienoate was injected i.p. at a dose of 1 mmol/kg body weight. The rats were killed 2 h after the treatment. The values are the means ± SD for the number of determinations

Tissue	Treatment	No. of determinations	H4-0	H4-1	H4-2	H4-3	H4-4
Hepatoma 7777	Control	9	49.2 ± 6.8	32.5 ± 5.6	6.3 ± 4.6	9.7 ± 7.8	2.3 ± 1.2
	CG1255	10	39.3 ± 7.3**	34.6 ± 4.9	12.5 ± 5.5*	11.5 ± 6.7	2.1 ± 1.2
Liver	Control	7	42.6 ± 2.9	41.8 ± 2.5	10.3 ± 2.3	0.8 ± 0.5	4.4 ± 1.6
	CG1255	8	39.1 ± 2.1*	44.1 ± 1.9	10.9 ± 1.0	1.2 ± 1.0	4.7 ± 1.8
Spleen	Control	6	47.0 ± 4.0	40.9 ± 3.1	8.0 ± 1.5	0.6 ± 0.4	3.5 ± 1.3
	CG1255	5	41.8 ± 4.9	44.1 ± 3.7	9.5 ± 1.9	0.9 ± 0.6	3.7 ± 1.4
Kidney	Control	6	53.9 ± 4.8	35.2 ± 3.8	7.7 ± 1.9	0.7 ± 0.7	2.6 ± 1.4
	CG1255	5	48.9 ± 1.8	39.8 ± 2.1*	8.1 ± 0.8	0.8 ± 0.5	2.3 ± 1.7

*$P < 0.05$, **$P < 0.01$, relative to control values

Table 9 Reversibility of the induction of H4 histone acetylation in DS19 mouse erythroleukemia cells incubated for 2 h with 5-phenylpenta-2,4-dienoate (CG-1255) at a concentration of 1 m*M* in 20 ml RPMI 1640 medium containing 25 m*M* HEPES and 5% fetal calf serum then resuspended in fresh medium and incubated for a further 6 h. The values shown are the percentages of unacetylated and acetylated H4 histones in relation to total H4 histone, and are the means ± SD for six to eight determinations

Incubation	Percent of total H4 histone				
	H4-0	H4-1	H4-2	H4-3	H4-4
Control	70.9 ± 7.6	21.2 ± 7.1	1.7 ± 1.4	1.9 ± 1.4	4.2 ± 3.9
CG1255 for 2 h	54.5 ± 9.4**	28.1 ± 4.2	8.1 ± 5.9*	7.0 ± 5.5*	2.4 ± 1.6
CG1255 for 2 h then fresh medium for 6 h	69.4 ± 4.1	25.0 ± 7.5	2.8 ± 4.0	1.4 ± 0.5	1.4 ± 1.0

*$P < 0.05$, **$P < 0.005$, relative to control values

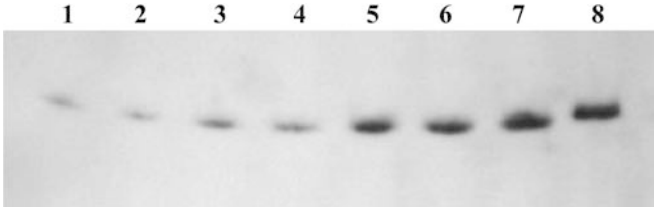

Fig. 1 Increased acetylation of uH2A histone induced by incubation of DS19 cells for 24 h with 5-phenyl-2,4-pentadienoate. Histones from control and treated cells were subjected to electrophoresis and an immunoblot was performed using an antibody against acetylated H2A histone. *Lane 1* histones from control cells. Concentrations of 5-phenyl-2,4-dienoate incubated with the cells: *lanes 2–4* 0.1 m*M*, *lanes 5 and 6* 0.3 m*M*, *lanes 7 and 8* 1.0 m*M*

Discussion

Inhibitors of histone deacetylase are being investigated as cancer chemotherapeutic agents [8, 12, 13, 19, 29]. Butyrate was the first inhibitor of histone deacetylase to be recognized [1, 24, 27] but the rapid metabolism of this compound has precluded its therapeutic application. Butyrate has generally been found to be a more potent inhibitor of histone deacetylase than other fatty acids of shorter or longer chain length. We have observed previously that phenylbutyrate is a more potent inhibitor of histone deacetylase than phenylacetate [15] and the present observations indicate that phenylbutyrate is

more effective than phenyl alkanoic acids having a chain length greater than four carbons. Both the chain length and the nature of the ring structure appear to be critical. Cyclohexyl butyrate was less effective than 4-phenylbutyrate both as an inhibitor of histone deacetylase activity and as an inhibitor of cell proliferation. In agreement with previous reports, we observed inhibition of cell proliferation by 4-phenylbutyrate in erythroleukemic cells [15], prostate cancer cells [2, 20, 22] and colon cancer cells [4, 6]. Evidence was obtained for these three cell types that greater inhibitory effects can occur with some phenyl alkanoic acids having longer side chains than 4-phenylbutyrate.

A variety of effects have been reported after treatment of cells with 4-phenylbutyrate in addition to the inhibition of histone deacetylase. It is not clear which are primary and which are secondary effects. 4-Phenylbutyrate has been reported to serve as a ligand for peroxisome proliferator-activated receptors (PPARs) alpha and gamma [23, 26]. Ligands of PPAR gamma have been reported to inhibit proliferation of several types of cancer cells [10, 25]. If the situation with phenyl alkanoic and phenyl alkenoic acids resembles that with fatty acids then one would anticipate that longer unsaturated chains would exhibit greater action as PPAR ligands than compounds with shorter saturated chains. Our data indicated a greater inhibition of growth with longer chain phenyl alkanoic acids and with unsaturated chains than with saturated chains. It appears possible, therefore, that the growth-inhibitory effect of the longer chain phenyl alkanoic acids might be mediated through action as PPAR gamma ligands.

In the case of 4-phenylbutyrate, it is possible that the growth-inhibitory effects might be mediated in part by both inhibition of histone deacetylase and action as a PPAR gamma ligand. The action may be further enhanced by combination with a more potent PPAR gamma agonist as in the study of Chang and Szabo [3] with a combination of 4-phenylbutyrate and ciglitizone. The interplay of histone deacetylase activity and PPAR gamma may be affected by their close association since there is evidence of a complex of PPAR gamma and histone deacetylase 3 with the RB protein [5]. In some systems bisphenol A diglycidyl ether (BADGE) has been found to act as a PPAR gamma antagonist. However, in preliminary studies with T47D human beast cancer cells and in PC3 and Caco-2 cells we have observed that BADGE at concentrations of 25 or 50 μM had an inhibitory effect on thymidine incorporation into DNA that was additive with the inhibitory effects of 4-phenylbutyrate, CG1255 and the PPAR gamma agonist ciglitizone [18]. We conclude that actions of phenyl alkanoic acids that may be mediated through PPAR gamma merit further investigation, particularly for compounds with longer side chains. The present results indicate that longer chain phenyl alkanoic acids are more potent inhibitors of cell proliferation than phenyl butyrate but may differ in their mechanism of action.

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