Induction of Differentiation of Colon Cancer Cells by Combined Inhibition of Kinases and Histone Deacetylase

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Abstract. *The MAP kinase pathway inhibitor U0126 in combination with butyrate promotes differentiation in some colon cancer cell lines. We examined several inhibitors of histone deacetylase (HDAC) in combination with U0126 and other protein kinase inhibitors to see if these effects are general properties of HDAC inhibitors or butyrate alone. Alkaline phosphatase and peptidase activities were examined as markers for cellular differentiation in the human colon cancer cell lines Caco-2 and HT29 and the minimally transformed NCM460. Several HDAC inhibitors caused greater increases of alkaline phosphatase in the cancer cells than in NCM460, in which butyrate was the only HDAC inhibitor that caused a consistent increase. Unlike the JNK and PKC inhibitors examined, the MEK 1/2 inhibitor U0126 induced alkaline phosphatase activity in Caco-2 as a single agent and caused additive effects with HDAC inhibitors. The PI-3 kinase inhibitor LY294002 had little effect alone but enhanced the response of most HDAC inhibitors as did the raf inhibitor GW5074. In addition to butyrate, several HDAC inhibitors can induce differentiation in colon cancer cells and the responses may be enhanced by U0126, GW5074 and LY294002.*

Sodium butyrate, an inhibitor of histone deacetylase activity, can cause induction of differentiation in colon cancer cells as judged by alkaline phosphatase activity (1-4). There is current interest in the combined action of histone deacetylase inhibitors and other anticancer agents. A study by Witt *et al.*, (5) indicated a synergistic action of butyrate and U0126, a MEK 1/2 inhibitor, in the induction of differentiation in HT29 human colon cancer cells. A similar combined action was observed by Orchel *et al.* (6) in Caco-2 human colon cancer cells. On the other hand, the effect of butyrate on alkaline phosphatase activity was significantly attenuated in the presence of inhibitors of protein kinase C and JNK. These observations prompted us to study several inhibitors of histone deacetylase in combination with U0126 and other protein kinase inhibitors in order to see if the effects on growth cessation and differentiation are general properties of histone deacetylase inhibitors or are restricted to the actions of butyrate alone. The inhibitors of histone deacetylase that were studied included some that are well established such as 4-phenylbutyrate (7, 8) and valproate (9) and others that have received little prior investigation including ethyl 2-mercaptoacetate, ethylene glycol bisthiogycolate, and 7-phenyl-2,4,6-heptatrienoylhydroxamic acid (CG1521). To obtain an indication of the specificity of the changes for transformed colon cells we compared the actions of drugs on Caco-2 and HT29 cells with effects on the minimally transformed human colon cell line, NCM460 (10).

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Materials and Methods

Cells and determination of cell proliferation. DS19 mouse erythroleukemia cells, HT29 and Caco-2 human colon cancer cells were incubated at 37˚C in RPMI 1640 medium with 5% fetal calf serum and 25 mM N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid) buffer. NCM460 human colon cells were maintained in M3:10 medium from INCELL Corp., San Antonio, TX, USA.

Reagents. 7-phenyl-2,4,6-heptatrienoylhydroxamic acid (CG1521) and ethylene glycol bisthiogycolate (BL1600) were provided by CircaGen Pharmaceutical, Phoenix, MD, USA.

Enzyme assays. 1.5 million cells were incubated with 10 ml RPMI 1640 medium with 25 mM HEPES and 5% fetal calf serum. The medium was changed after 24 hours and unless stated otherwise the cells were incubated for 72 hours before harvesting. The cells

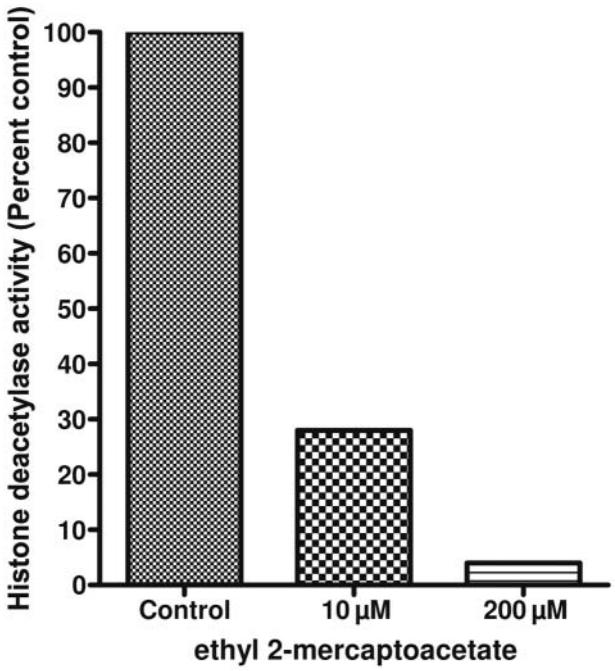

Figure 1. *Inhibition of histone deacetylase activity by ethyl 2-mercaptoacetate.*

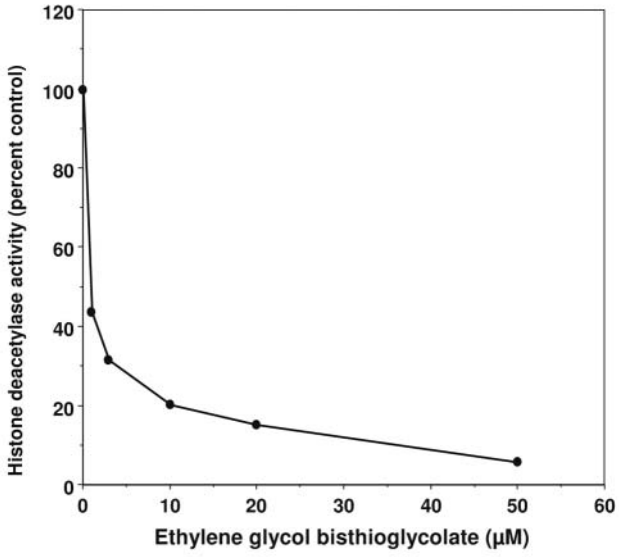

Figure 2. *Inhibition of histone deacetylase activity by ethylene glycol bisthioglycolate (BL1600).*

were washed with phosphate buffered saline and extracted with 0.5% NP40, 0.25 M NaCl, 5 mM EDTA and 50 mM Tris pH 8.0. The protein concentration of the extract was determined using the BCA Protein Assay Reagent from Pierce, Rockford, IL, USA. Enzymes were assayed at 37˚C. Alkaline phosphatase was assayed in a volume of 0.22 ml using para-nitrophenyl phosphate (4.5 mM) as substrate and 6.8 mM 2-amino-2-methyl-1-propanol pH 10.3 as the buffer. Incubations were for 30 minutes and were stopped by the addition of 2.0 ml 0.05 N NaOH. Formation of product was monitored by the change in absorbance at 410 nm. Aminopeptidase and dipeptidyl peptidase were assayed in a volume of 0.5 ml using 2 mM L-alanyl-4-nitroanilide or 2 mM Gly-Pro-4-nitroanilide as substrates, respectively. The procedure was based on the method of Nagatsu *et al.* (12). The buffer was 0.1 M Tris pH 8.0. Incubations were stopped by the addition of 2.0 ml 1 M acetate pH 4.2. Production of the product, 4-nitroaniline, was monitored at 405 nm.

Histone acetylation and deacetylase activity. The isolation of histones and electrophoresis on urea-acetic acid polyacrylamide gels was performed as previously described (8). The relative levels of acetylated H4 histones were quantitated by densitometry of Coomassie-blue-stained gels. Histone deacetylase activity was measured by the release of tritium-labeled acetate from histones in nuclei of DS19 mouse erythroleukemia cells as previously reported (7).

Statistical evaluation. Statistical significance of the results was determined by a two-tailed Student's *t*-test or by Dunnett's test for multiple comparisons using the Instat program. A probability of less than 5% was considered significant.

Results

The data in Figures 1 and 2 show that the sulfhydryl compounds ethyl 2-mercaptoacetate and ethylene glycol bisthioglycolate are inhibitors of histone deacetylase activity. Compounds that are inhibitors of histone deacetylase cause an accumulation of acetylated histones and we have confirmed that this occurs with the compounds ethyl 2-mercaptoacetate, ethylene glycol bisthioglycolate and 7-phenyl-2,4,6-heptatrienoylhydroxamic acid (data not shown).

As an extension of literature studies on the combined action of U0126 and butyrate on the induction of alkaline phosphatase in colon cancer cells we examined the combined effects of U0126 and 4-phenylbutyrate. Additivity was seen with Caco-2 cells (Figure 3A) and HT29 cells (Figure 3B) but not with the NCM460 colon cells (Figure 3C). Although we observed that alkaline phosphatase activity could be induced by 0.5-1.0 mM butyrate, several inhibitors of histone deacetylase caused a decrease in alkaline phosphatase activity in NCM460 cells. This was seen with ethyl 2-mercaptoacetate (Figure 4), ethylene glycol bisthioglycolate (Figure 5) and CG1521 (Figure 6) and contrasts with the induction of alkaline phosphatase observed in Caco-2 colon cancer cells.

In Caco-2 cells, additive or synergistic induction of alkaline phosphatase was seen after treatment with a combination of U0126 and several inhibitors of histone deacetylase including ethyl 2-mercaptoacetate (Figure 7), ethylene glycol bisthioglycolate (Figure 8) and valproate (Figure 9A). Growth inhibitory effects were indicated by decreased protein yield in extracts of cells treated with

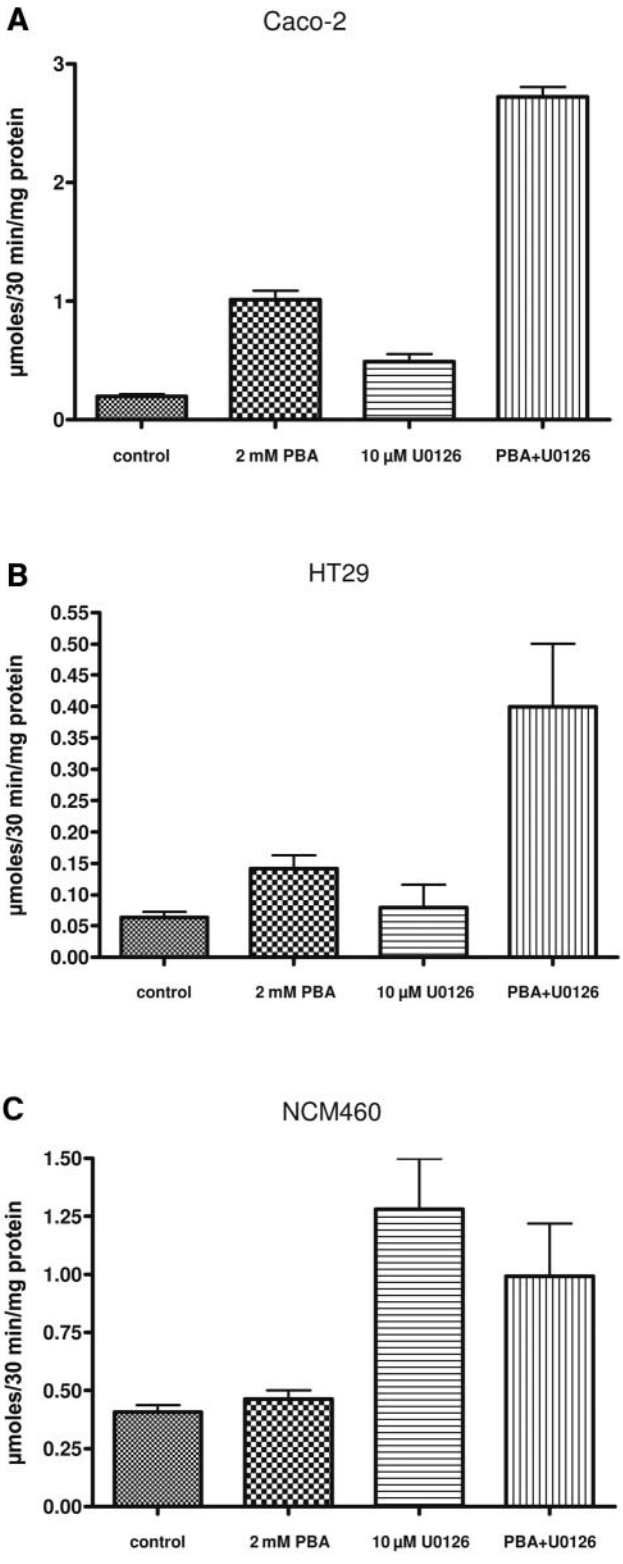

Figure 3. *Effect of incubation with 10 µM U0126 and 2 mM 4-phenylbutyrate for 72 hours on alkaline phosphatase activity in (A) Caco-2 cells, (B) HT29 cells and (C) NCM 460 cells.*

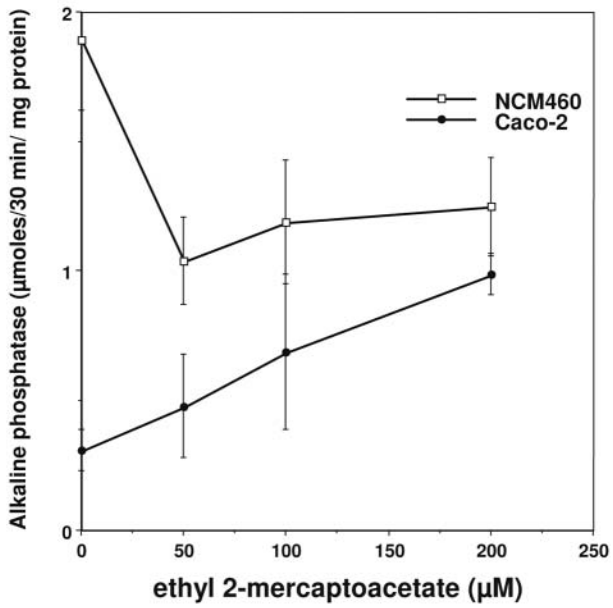

Figure 4. *Effect of incubation with ethyl 2-mercaptoacetate for 72 hours on the activity of alkaline phosphatase in NCM460 and Caco-2 cells.*

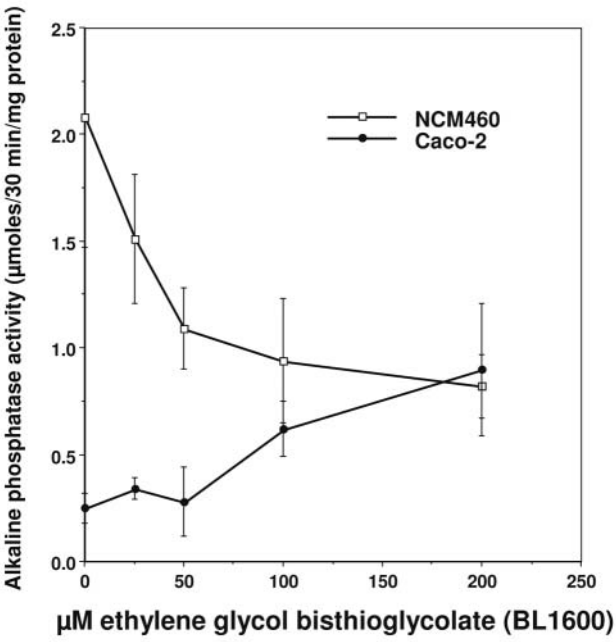

Figure 5. *Effect of incubation with ethylene glycol bisthioglycolate for 72 hours on the activity of alkaline phosphatase in NCM460 and Caco-2 cells.*

combinations of U0126 and HDAC inhibitors. For example, in the groups presented in Figure 7, the mean protein yield relative to controls for cells incubated with 10 µM U0126, 50 µM ethyl 2-mercaptoacetate, and the combination of 10 µM U0126 and 50 µM ethyl 2-

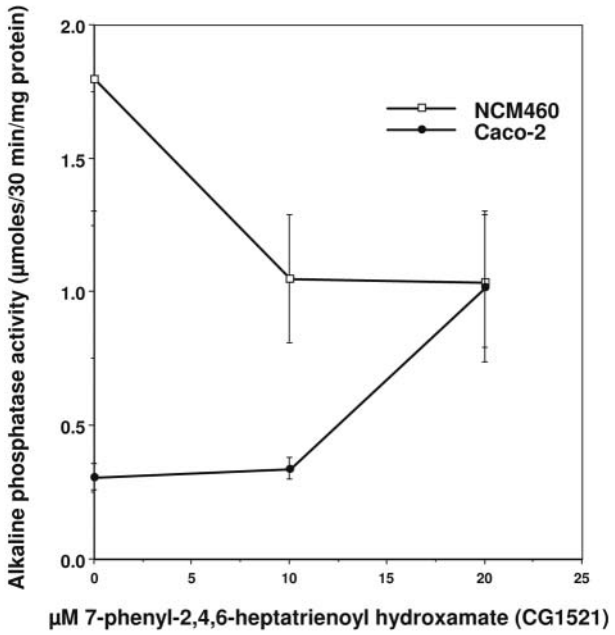

Figure 6. *Effect of incubation with 7-phenyl-2,4,6-heptatrienoylhydroxamic acid for 72 hours on the activity of alkaline phosphatase in NCM460 and Caco-2 cells.*

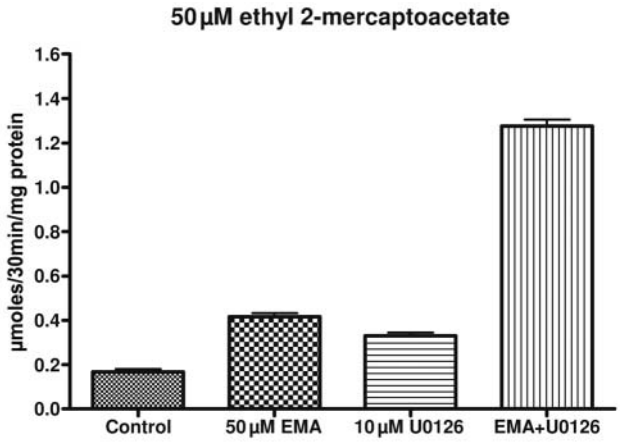

Figure 7. *Induction of alkaline phosphatase activity by incubation of Caco-2 cells for 72 hours with 50 µM ethyl 2-mercaptoacetate (EMA) and 10 µM U0126.*

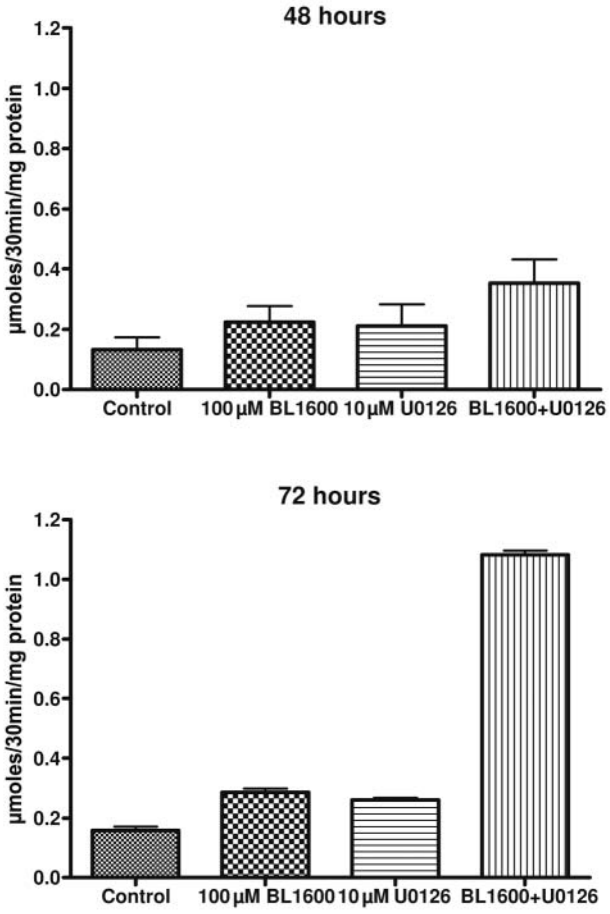

Figure 8. *Induction of alkaline phosphatase activity by incubation of Caco-2 cells for 48 or 72 hours with 100 µM ethylene glycol bisthioglycolate (BL1600) and 10 µM U0126.*

mercaptoacetate was 84%, 74% and 54%, respectively. The data in Figure 9A and B indicate that the fold induction of alkaline phosphatase by U0126 and valproate in Caco-2 cells is greater than for dipeptidyl peptidase. The greater induction of alkaline phosphatase after 72 hours than after 48 hours was seen in other preliminary studies and prompted us to generally use a 72-hour incubation period in these investigations.

We confirmed the observation of Orchel *et al.* (6) that the induction of alkaline phosphatase by butyrate in Caco-2 cells was not enhanced by co-incubation with SP600125, a JNK inhibitor, or Ro-31-8220, a protein kinase C inhibitor. Furthermore there was no enhanced induction of alkaline phosphatase when these protein kinase inhibitors were combined with HDAC inhibitors other than butyrate including ethyl 2-mercaptoacetate, 4-phenylbutyrate and trichostatin A (data not shown). In addition we have not seen enhanced induction of alkaline phosphatase activity in combination with HDAC inhibitors and some other protein kinase inhibitors including H89, a protein kinase A inhibitor, SB203580, an inhibitor pf p38 protein kinase, and two AKT inhibitors, triciribine and AKT inhibitor IV (data not shown).

In early passages of NCM460 cells the activity of alkaline phosphatase was higher than in Caco-2 cells while the activity of dipeptidyl peptidase was lower. However, in later

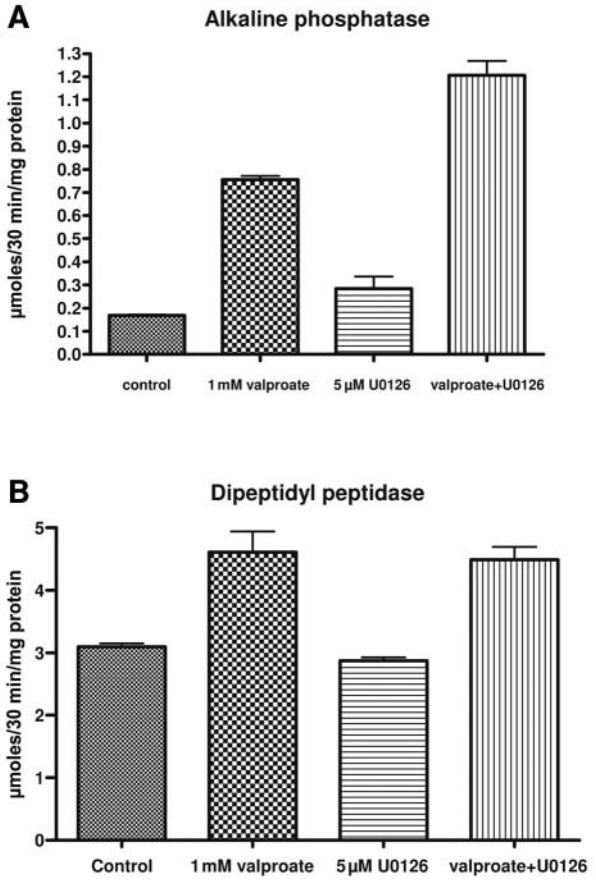

Figure 9. *Effect of incubation of Caco-2 cells for 72 hours with 1 mM valproate and 5 µM U0126 on the activity of (A) alkaline phosphatase and (B) dipeptidyl peptidase.*

Table I. *Percentage of unacetylated histone H4 in Caco-2 cells after incubation with butyrate, U0126 and SP600125.*

Treatment	Unacetylated H4 histoneas as a percentage of total H4 histone
Control	55.7%
1 mM butyrate	13.0%
10 µM U0126	55.6%
10 µM U0126 + 1 mM butyrate	11.7%
10 µM SP600125	65.2%
10 µM SP600125 + 1 mM butyrate	8.6%

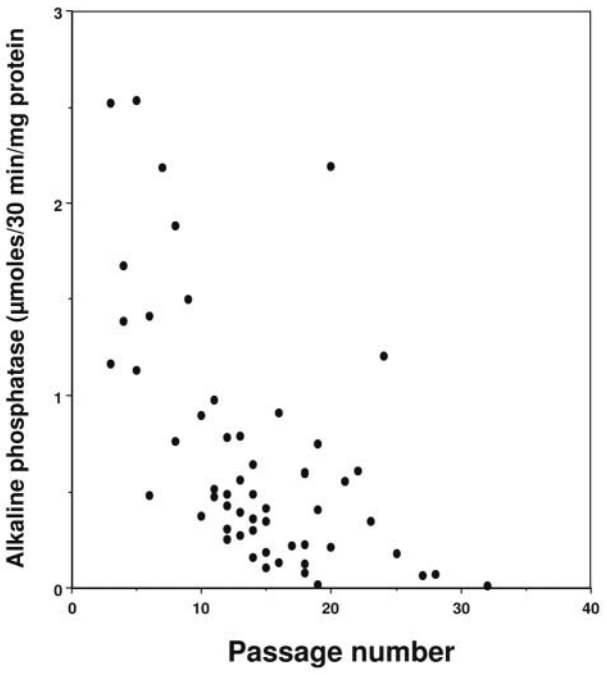

Figure 10. *Decrease in alkaline phosphatase activity in NCM460 cells with increasing passage number.*

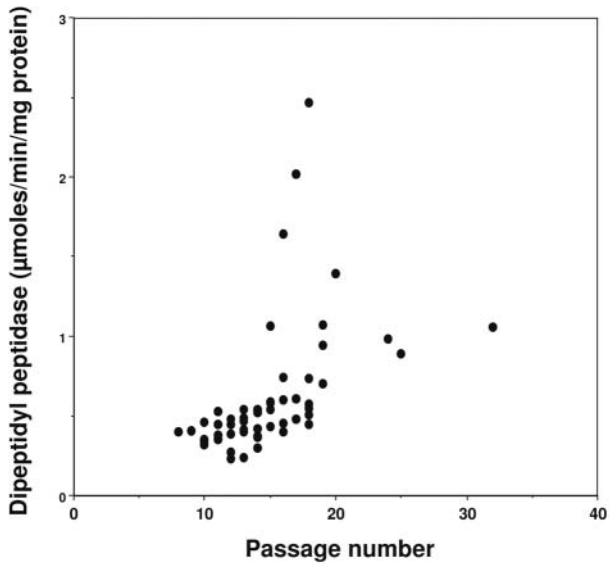

Figure 11. *Increase in dipeptidyl peptidase activity in NCM460 cells with increasing passage number.*

passages there was a decline in alkaline phosphatase activity (Figure 10) and an increase in dipeptidyl peptidase activity (Figure 11). In studying the combined action of U0126 and inhibitors of histone deacetylase activity we considered that U0126 might modify the acetylation level of histones. However, the data in Table I indicate that 10 µM U0126 did not modify the level of H4 acetylation in Caco-2 cells and did not affect the level of histone acetylation induced by treatment with butyrate as indicated by the decrease in unacetylated histone H4.

Alkaline phosphatase activity can be induced in NCM460 cells by incubation with butyrate and U0126 either as single

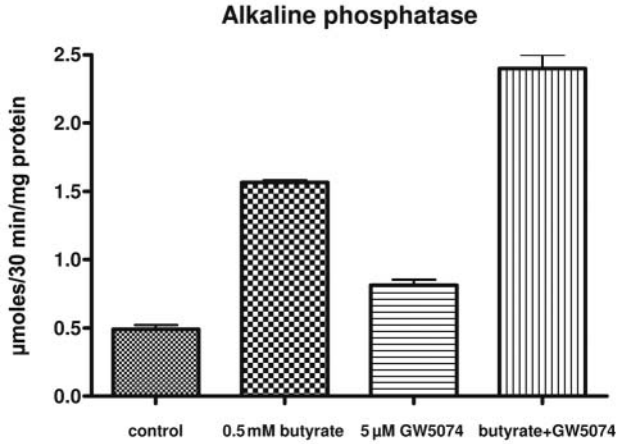

Figure 12. *Induction of alkaline phosphatase activity by incubation of NCM460 cells for 72 hours with 0.5 mM butyrate and 5 µM GW5074.*

agents or in combination, and this prompted us to examine the action of an agent that acts as an inhibitor upstream of U0126 in the MAPK pathway, namely the raf-inhibitor, GW5074. As shown in Figure 12, there was an additive effect of butyrate and GW5074 on the induction of alkaline phosphatase in NCM460 cells. Additive effects of 5 µM GW5074 and either 0.5 mM butyrate or 0.5 mM valproate were also seen for the induction of alkaline phosphatase in Caco-2 cells (data not shown).

Synergistic induction of alkaline phosphatase in Caco-2 cells was seen after combined incubation with butyrate and the PI3 kinase inhibitor LY294002 (Figure 13A). Much smaller changes were seen in dipeptidyl peptidase activity and there was no induction of aminopeptidase activity (Figure 13 B and C). There was no significant induction of alkaline phosphatase when cells were treated with LY294002 as a single agent.

Discussion

Alkaline phosphatase activity has been used as a marker for differentiation in many studies with Caco-2 and HT29 colon cancer cells but we are not aware of other studies of alkaline phosphatase activity in the NCM460 colon cells. The NCM460 cell line was first described as an untransformed colon cell (10) but studies by us and other investigators have indicated that there can be changes in the character of this cell line with continued passage in culture. The high alkaline phosphatase activity in early passages of NCM460 cells and the decline in later passages may imply a loss of differentiated function and was accompanied by morphological changes that made the cells more similar to the HT29 and Caco-2 cells. In all three cell lines there was induction of alkaline phosphatase activity by butyrate and

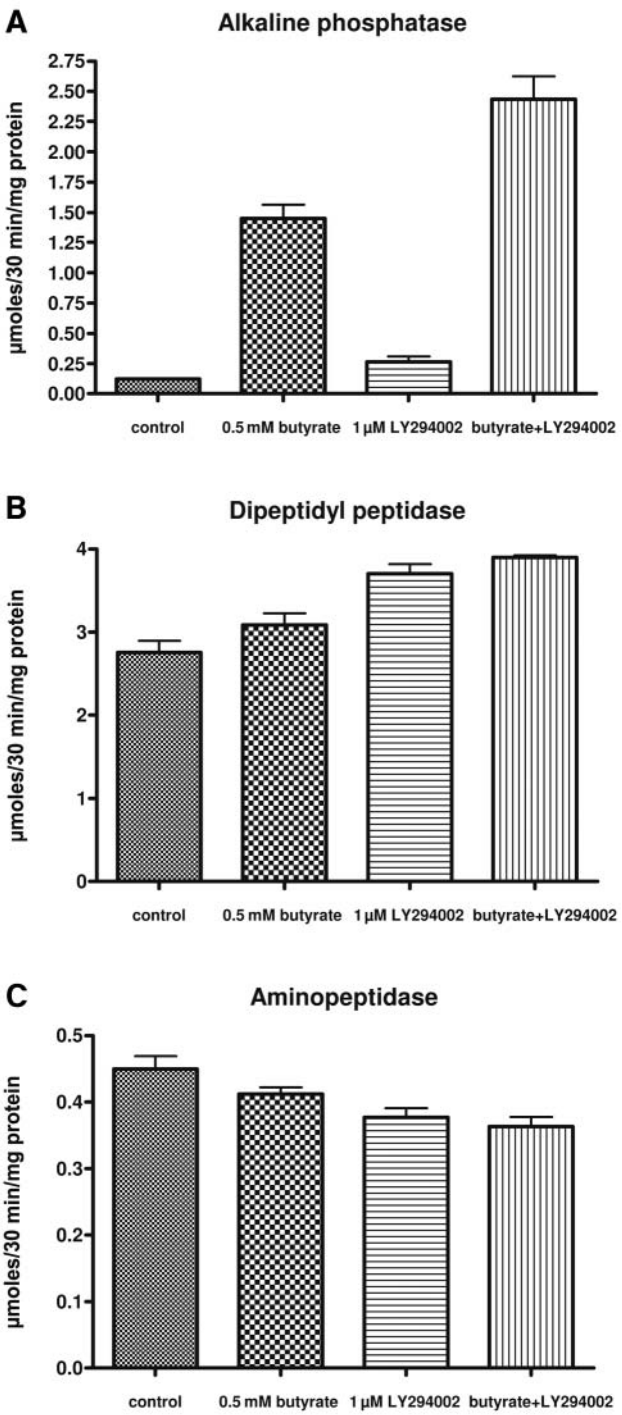

Figure 13. *Effect of incubation of Caco-2 cells for 72 hours with 0.5 mM butyrate and 1 µM LY294002 on the activity of (A) alkaline phosphatase, (B) dipeptidyl peptidase and (C) aminopeptidase.*

the MEK1/2 inhibitor U0126. However, the NCM460 cells were less responsive to inhibitors of histone deacetylase activity other than butyrate, including 4-phenylbutyrate,

valproate, ethyl 2-mercaptoacetate and ethylene glycol bisthioglycolate and the hydroxamate CG1521. 4-Phenylbutyrate had small effects that could be positive or negative whereas HDAC inhibitors other than butyrate and 4-phenylbutyrate consistently caused decreases in specific activity.

Our identification of dipeptidyl peptidase and aminopeptidase activity was based on the published substrate specificity of these enzymes. Thus, dipeptidyl peptidase has been reported not to hydrolyze Ala 4-nitroanilide (13). We have designated activity with Ala 4-nitroanilide as aminopeptidase activity although some reports in the literature have attributed activity with Ala 4-nitroanilide to dipeptidyl dipeptidase (14). In our studies, aminopeptidase activity with Ala 4-nitroanilide as a substrate showed only limited response to the agents studied and tended to serve as a control enzyme relative to the frequently much greater responses of alkaline phosphatase and, to a lesser extent, dipeptidyl peptidase. The increased dipeptidyl peptidase activity that we observed after incubation with butyrate was less than the increases that have been observed with other conditions for the induction of differentiation of Caco-2 and HT29 cells including prolonged culture and glucose-free medium (15).

Synergistic effects on cell death in tumor cells have been reported for inhibitors of the MAP kinase pathway and HDAC inhibitors (16-19). Growth inhibitory effects in our studies were reflected by decreased protein yield in extracts of cells treated with combinations of U0126 and HDAC inhibitors. Orchel *et al.* (6) did not observe induction of alkaline phosphatase activity in Caco-2 cells treated for 48 hours with U0126 as a single agent but there was synergistic induction of alkaline phosphatase when co-incubated with butyrate. We confirmed the synergistic action at 48 hours but observed that after 72 hours there was also significant induction of alkaline phosphatase with U0126 as a single agent.

Augmentation of apoptosis has also been recorded for a combination of an HDAC inhibitor and phophatidylinositol 3-kinase inhibition (20, 21). At dose levels where viability was maintained, we have seen a synergistic induction of alkaline phosphatase activity in Caco-2 cells when treated with a combination of LY294002 and butyrate or other HDAC inhibitors.

There is much current interest in combination therapy of cancer with HDAC inhibitors and other agents. The present studies add support to the concept that combinations with inhibitors of the MAP kinase and the phophatidylinositol 3-kinase signaling pathways merit further investigation.

Acknowledgements

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