

SHORT REPORT

PHENYLBUTYRATE INHIBITS THE INVASIVE PROPERTIES OF PROSTATE AND BREAST CANCER CELL LINES IN THE SEA URCHIN EMBRYO BASEMENT MEMBRANE INVASION ASSAY

Erica S. DYER¹, Michelle T. PAULSEN¹, Sonja M. MARKWART¹, Meidee GOH¹, Donna L. LIVANT¹ and Mats LJUNGMAN^{1,2*}

¹Department of Radiation Oncology, Division of Radiation and Cancer Biology, University of Michigan Comprehensive Cancer Center, University of Michigan Medical School, Ann Arbor, MI, USA

²Department of Environmental Health Sciences, School of Public Health, University of Michigan, Ann Arbor, MI, USA

Histone deacetylase inhibitors, such as phenylbutyrate, are currently undergoing clinical trials as potential anticancer agents. Phenylbutyrate can induce cell differentiation and apoptosis in a number of cancer cell types and can act in synergy with ionizing radiation and chemotherapy to induce apoptosis. We used the sea urchin embryo basement membrane invasion assay to show that phenylbutyrate potently inhibited the invasive properties of both prostate and breast cancer cells at clinically achievable doses. This inhibition was dose-dependent and persisted for at least 24 hr after the drug was removed. These results suggest that in addition to activating apoptosis in cancer cells, phenylbutyrate may be used in prevention of metastatic disease.

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A number of clinical trials are currently ongoing to evaluate the efficacy of histone deacetylase (HDAC) inhibitors in the treatment of cancer.^{1,2} Phenylbutyrate is an HDAC inhibitor that is odorless and orally active and has shown promising results in Phase I and Phase II clinical trials.¹ As an HDAC inhibitor, phenylbutyrate induces hyperacetylation of chromatin leading to changes in gene expression^{2,3} and the induction of differentiation and apoptosis.^{4–7} It has been shown that butyrate and phenylbutyrate can act in synergy with radiation^{8–11} or chemotherapy^{11,12} to induce cells to undergo apoptosis. Furthermore, some HDAC inhibitors have recently been found to inhibit angiogenesis.¹³ The anti-angiogenic properties of HDAC inhibitors may occur through the down regulation of VEGF expression.¹⁰

In our previous study we found that phenylbutyrate reduced the expression of caveolin-1.¹⁰ Although the exact role of caveolin-1 in carcinogenesis is unclear, caveolin-1 is thought to regulate integrin signaling¹⁴ and high expression of caveolin-1 is associated with a metastatic phenotype in breast and prostate cancer.^{15–17} In addition to reducing caveolin-1 expression, phenylbutyrate has been found to reduce the expression of the invasion-related plasminogen activator, urokinase, in glioma cells¹⁸ and reduce the invasiveness of cancer cells in artificial matrigel chamber invasion assays.^{18,19}

We explored the ability of phenylbutyrate to block the invasiveness of metastatic prostate and breast cancer cells using the sea urchin embryo basement membrane invasion assay. This invasion assay utilizes the natural occurring, selectively permeable basement membranes of sea urchin embryos (SU-ECM) as invasion substrates.^{20,21} The sea urchin embryo basement membrane is functionally analogous to the mammalian basement membranes underlying the epidermis because it is invaded by pigment cells during development.²² We have shown previously that these SU-ECM invasion substrates can be selectively invaded by metastatic tumor cells.²⁰ In fact, we have shown that this assay can predict the metastatic potential of cancer cell lines with greater than 99% confidence.²⁰ We show that phenylbutyrate effectively inhibits the invasiveness of metastatic prostate and breast cancer cells in the SU-ECM invasion assay.

MATERIAL AND METHODS

Cell cultures and phenylbutyrate treatments

PC3 and DU-145 cells were obtained from American Type Culture Collection (Manassas, VA) and Steve Ethier, University of Michigan, kindly provided us with the SUM-149 cell line. The PC3 and DU-145 cells were cultured in MEM (Life Technologies, Grand Island, NY) supplemented with 10% FBS (HyClone, Logan, UT), with 1× amino acids, vitamins and antibiotics. SUM-149 cells were grown in Ham's F-12 media supplemented with 5% FBS, insulin (5 µg/ml), hydrocortisone (2 µg/ml), gentamicin (5 µg/ml) and fungizone (2.5 µg/ml). All cells were maintained at 37°C in 5% CO₂. The maintenance of the sea urchin embryos cultures and the isolation of the sea urchin embryo basement membranes (SU-ECM) were carried out as described previously.^{20,21} Phenylbutyrate was purchased from Sigma-Aldrich (St. Louis, MO) and was dissolved in either benzene in a 1 M stock solution or in 10× PBS in a 50 mM stock solution. Stock solutions of phenylbutyrate were diluted to appropriate concentrations using culture media and the cells (≈70% confluence) were treated for 24 hr.

Invasion assay

After a 24-hr treatment with phenylbutyrate, the cells were analyzed for their invasiveness using the sea urchin embryo basement membrane invasion assay as described previously.^{20,21} The media containing phenylbutyrate were aspirated and the cells were washed with PBS. The cells were then isolated using a brief trypsin treatment, collected by centrifugation and washed once with PBS and centrifuged. The cells were then resuspended to a concentration of 20,000 cells/ml in the appropriate media containing either 10% FBS for the DU-145 and PC3 cells or 5% FBS for the SUM-149 cells. The cells were then layered on top of the embryo basement membrane invasion substrates and incubated for 4 hr at 37°C. Relative invasion was scored by phase contrast microscopic examination. For each sample, 50–100 SU-ECM were examined and the ratio of cancer cells located inside *vs.* adhering to the outside of the SU-ECMs were calculated. The invasion percentages of the control cells ranged between 12–15% and were in the figures set as 100% relative invasiveness.

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*Correspondence to: Department of Radiation Oncology, University of Michigan Comprehensive Cancer Center, 4306 CCGC, 1500 E. Medical Center Drive, Ann Arbor, MI 48109-0936. Fax: +1-734-647-9654. E-mail: ljungman@umich.edu

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Measurements of apoptosis and clonogenic survival

For apoptotic cell examinations, cells were treated with different concentrations of phenylbutyrate for 48 hr and both floating and attached cells were then collected and analyzed for percentages of sub-G1 DNA containing cells by flow cytometry as described previously.²³ For clonogenic survival measurements, cells were treated with different concentrations of phenylbutyrate for 24 hr after which the cells were rinsed, trypsinized and counted. A known number of cells were then plated in fresh media and 10 days later the cells were fixed, stained and colonies counted under the microscope.

RESULTS

Phenylbutyrate inhibits invasiveness of metastatic prostate and breast cancer cells

We had shown previously that phenylbutyrate attenuated the expression of caveolin-1 in prostate cancer cells.¹⁰ Because caveolin-1 is thought to regulate integrin signaling pathways and has been shown to correlate with the metastatic potential of prostate cancer cells^{14–17} we decided to investigate the effects of phenylbutyrate treatment on the invasiveness of metastatic prostate and breast cancer cells using the sea urchin embryo basement membrane (SU-ECM) invasion assay.²⁰ This invasion assay has the advantages over the matrigel invasion assay in that it uses naturally occurring invasion substrates consisting of a sea urchin embryo basement membranes that are very uniform and functionally resemble human basement membranes that are invaded during the metastatic stage of cancer.²⁰ Furthermore, this assay can predict with greater than 99% confidence the metastatic potential of a cancer cell line.²⁰

We used the metastatic prostate cancer cell lines PC3 and DU-145 because they have been shown to efficiently invade SU-ECM substrates.²¹ We found that a pretreatment of the cells for 24 hr with 2 mM phenylbutyrate markedly inhibited the ability of these cells to invade the SU-ECM (Fig. 1). We also used the metastatic breast

cancer cell line SUM-149 and found that these cells readily invaded the SU-ECM. Similarly to the prostate cancer cell lines, when the breast cancer cells had been pretreated with phenylbutyrate they completely lost their ability to invade the SU-ECM (Fig. 1).

Phenylbutyrate inhibits invasiveness in a dose-dependent manner

We next investigated the dose response of phenylbutyrate on the inhibition of invasion of SU-ECM. We pretreated DU-145 prostate cancer cells for 24 hr with doses of phenylbutyrate ranging from 0.1–2.0 mM before testing their invasiveness. The results show that phenylbutyrate inhibits invasion in a dose-dependent manner (Fig. 2). Invasiveness was reduced to 54%, 24% and 13% compared to mock-treated control cells when cells had been pretreated with 0.1 mM, 0.5 mM and 2.0 mM, respectively.

Blockage of invasion by phenylbutyrate is not readily reversible

In the clinical setting, continuous treatment of patients with chemotherapeutic drugs is often not feasible. Consequently, the effects of the drugs may rapidly diminish after termination of treatment. To investigate whether the inhibitory effect of phenylbutyrate on the invasiveness of metastatic cancer cells is transient or would persist even 24 hr after the drug had been removed from the cells, we chased phenylbutyrate-treated cells with fresh media for 24 hr before analyzing invasiveness. The results show that incubation for 24 hr in drug-free media after 24 hr of phenylbutyrate treatment did not diminish the inhibition of invasion (Fig. 3). In fact, we observed an even greater inhibition for the chased cells than for the cells tested directly after phenylbutyrate treatment. Thus, the inhibitory effects of phenylbutyrate on invasion appear to be persistent for at least 24 hr after drug removal.

Inhibition of invasion is not explained by loss of cell survival

The mechanism by which phenylbutyrate inhibited invasion was not merely due to the toxicity of the phenylbutyrate treatment,

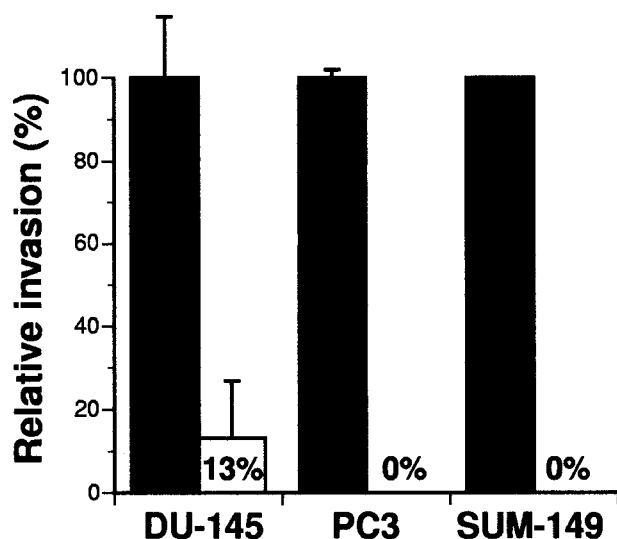


FIGURE 1 – Phenylbutyrate effectively inhibits the invasiveness of metastatic prostate and breast cancer cells in the SU-ECM invasion assay. Cells were pre-treated with 2 mM phenylbutyrate for 24 hr before being washed, trypsinized and subjected to the SU-ECM invasion assay. The data is presented as the relative percentage of cells invading the SU-ECM and represents the average of 2–6 independent experiments. Error bars = \pm SEM. The actual invasion percentage of untreated control cells were 14.5 ± 2.1 ($n = 6$), 13.1 ± 1.25 ($n = 2$) and 12.5 ± 0 ($n = 2$) for DU-145, PC3 and SUM-149, respectively. The data for the DU-145 cells was pooled from all the experiments shown ($n = 6$).

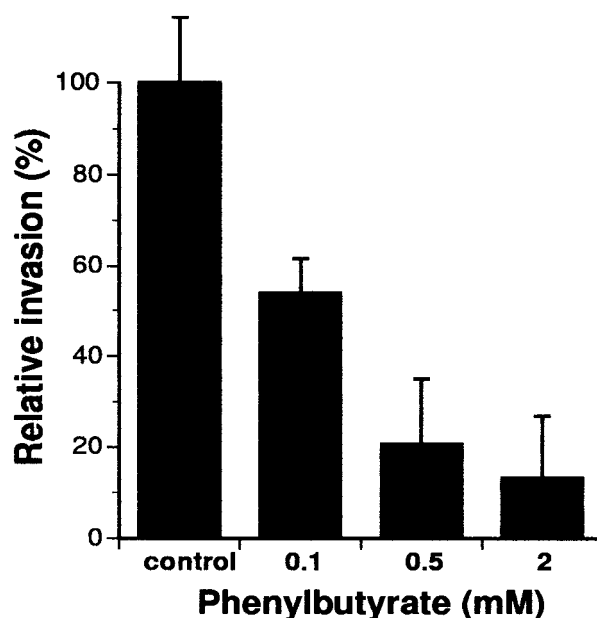


FIGURE 2 – Phenylbutyrate inhibits invasiveness in a dose-dependent manner. DU-145 cells were pretreated with different doses of phenylbutyrate before the cells were washed, trypsinized and subjected to the SU-ECM invasion assay. The data is presented as the relative percentage of cells invading the SU-ECM and represents the average of 2–6 independent experiments. Error bars = \pm SEM. The data for the control and 2 mM dose is pooled from all the experiments shown ($n = 6$).

because we observed that the cells that “missed” the invasion substrates readily attached and spread out at the bottom of the wells. To further evaluate the effect phenylbutyrate have on cell survival we treated cells with increasing doses of phenylbutyrate and measured either the induction of apoptosis or loss of clonogenic survival (Fig. 4). Only modest increases in apoptosis or loss of clonogenic survival were observed after treatments with phenylbutyrate. Thus, these results suggest that the inhibitory effect of phenylbutyrate on the invasion of the SU-ECM found in our study can not be explained by loss of viability.

DISCUSSION

Using the sea urchin embryo basement membrane (or SU-ECM) invasion assay, we showed that the HDAC inhibitor phenylbutyrate markedly inhibited the invasiveness of metastatic prostate and breast cancer cells. This inhibition was dose-dependent with about 50% inhibition observed at doses as low as 100 μ M and complete inhibition observed for some cancer cell lines at 2 mM. Furthermore, the inhibition of invasiveness was found to persist for at least 24 hr after drug removal suggesting that the inhibitory effect of phenylbutyrate was not transient. These results are in concordance with previous studies showing that HDAC inhibitors can inhibit the ability of metastatic cells to invade artificial matrigel invasion substrates.^{18,19} The present study using naturally occurring sea urchin embryo basement membranes showed a more dramatic inhibition compared to previous studies using artificial matrigel invasion chambers, suggesting that the potential inhibitory effect of phenylbutyrate on invasiveness of cancer cells *in vivo* may be even greater than previously thought.

The mechanism by which phenylbutyrate inhibits the invasive properties of metastatic cancer cells may involve HDAC-inhibi-

tion-mediated alterations of gene expression. A number of genes with proposed functions in the invasion process have been shown to have altered expression after treatment with HDAC-inhibitors. Caveolin-1, MMP-2 and urokinase have been found to be down-regulated,^{10,18,24,25} whereas the MMP inhibitors TIMP-1 and TIMP-2 are upregulated by butyrate.²⁶ Although some apoptosis has been observed in cancer cells after long exposures to 2 mM phenylbutyrate,^{5,7,12} we do not believe that induction or commitment to apoptosis is responsible for the loss of invasiveness of these cells after 24-hr incubations with phenylbutyrate. First, phenylbutyrate-treated cells appeared viable by microscopic evaluation and were able to attach to and spread out on the surface in the bottom of the wells similarly to the untreated cells. Second, cells treated for 48 hr showed only a modest increase in the induction of

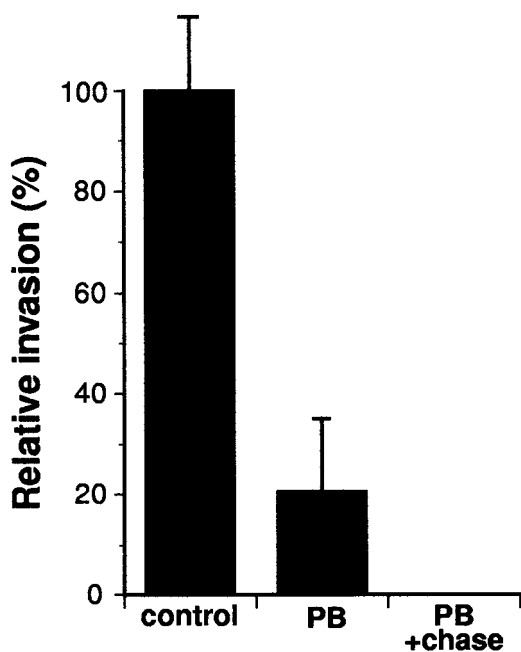


FIGURE 3—Inhibition of invasiveness by phenylbutyrate is not readily reversible. DU-145 cells were pretreated with 2 mM phenylbutyrate before the cells were washed and incubated for an additional 24 hr in drug-free media (PB + chase). The cells were then trypsinized and subjected to the SU-ECM invasion assay. The data is presented as the relative percentage of cells invading the sea urchin embryo substrates and represents the average of 2–6 independent experiments. Error bars = \pm SEM. The data for the control and phenylbutyrate (PB)-treated cells is pooled from all the experiments shown ($n = 6$).

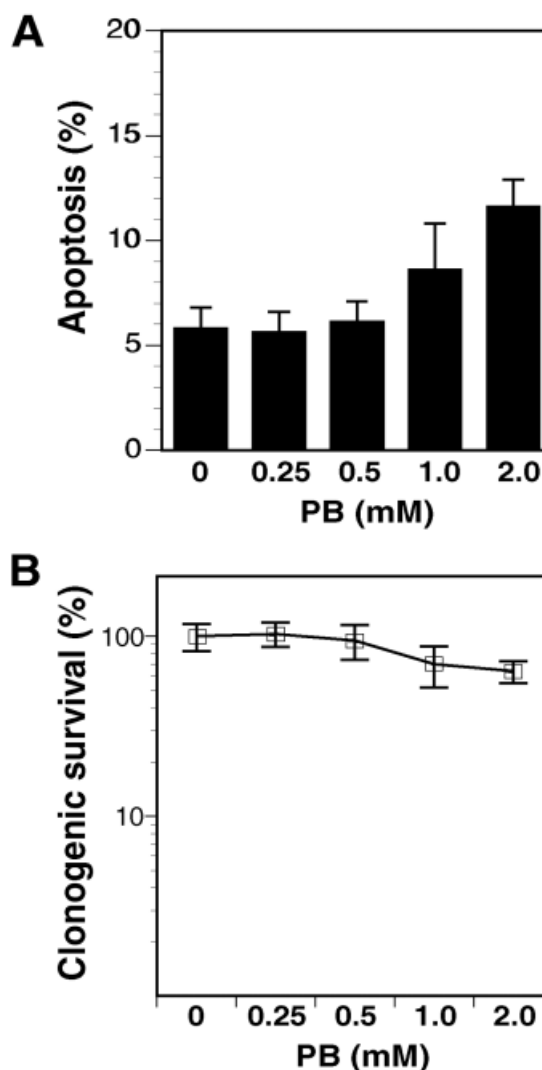


FIGURE 4—Modest induction of apoptosis or loss of clonogenic survival by phenylbutyrate. (a) DU145 prostate cancer cells were treated with different concentrations of phenylbutyrate for 48 hr followed by flow cytometric analysis of the percentages of sub-G1 DNA containing cells. The bars represent the mean of 4 biological experiments. Error bars = sample SD. (b) PC3 prostate cancer cells were treated for 24 hr with different concentrations of phenylbutyrate before known numbers of cells were seeded and allowed to grow into colonies for ten days. The values represent the mean of 5 different biological experiments done in triplicates ($n = 15$). Error bars = sample SD.

apoptosis. Significant apoptosis measured as sub-G1 DNA containing cells did not occur until after 72–96 hr of treatment with the higher doses (data not shown). Third, only a modest reduction in clonogenic survival was observed after a 24-hr treatment with phenylbutyrate.

The findings that prolonged treatments with phenylbutyrate can induce apoptosis directly in cancer cells^{5,7,12} and acts in synergy with radiation or chemotherapy to induce apoptosis^{10,12} makes phenylbutyrate very attractive as a potential anti-cancer agent. The findings in our study that phenylbutyrate effectively inhibits invasiveness of metastatic cancer cells at doses that can be safely

achieved in patients^{1,27} points to a new exciting possibility that phenylbutyrate may be useful as an anti-cancer agent in targeting the invasion phase of metastatic cancers.

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