Comparative Effects of 4-Phenyl-3-Butenoic Acid and Vorinostat on Cell Growth and Signaling

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Abstract. *Background/Aim: 4-phenyl-3-butenoic acid (PBA) is a small-molecule anti-inflammatory agent, which has been shown to inhibit growth, increase gap junction intercellular communication and modulate activation of p38 mitogen-activated protein kinase (p38 MAPK) and c-jun n-terminal kinase (JNK) in tumorigenic cells at concentrations that do not similarly affect non-tumorigenic cells. Vorinostat is an anticancer agent structurally similar to PBA. The purpose of this study was to compare the effects of these two agents on JNK and p38 activation, cell growth and gap junction intercellular communication (GJIC). Materials and Methods: Cell growth, GJIC and western blot analyses were performed utilizing tumorigenic WBras1 and H2009 human carcinoma cells, and non-tumorigenic WBneo3 and human bronchial epithelial (HBE) cells. Results: Both compounds significantly inhibited WBras1 and H2009 tumorigenic cell growth and increased GJIC in WBras1 cells, as previously reported for PBA. Under similar conditions, both compounds increased phosphorylation of p38 MAPK in tumorigenic but not in non-tumorigenic cells and decreased phosphorylation of JNK in tumorigenic cells. However, a decrease in phosphorylation of JNK occurred in non-tumorigenic WBras1 cells following vorinostat treatment but not PBA treatment. Both compounds showed a selective growth inhibition of H2009 human carcinoma over normal HBE lung cells but, unlike PBA, vorinostat significantly decreased cell growth in WBneo3 cells. Conclusion: Overall, PBA exhibited similar effects to vorinostat in tumorigenic cells, while also showing reduced effects on JNK phosphorylation and growth in non-tumorigenic cells compared to ras-transformed cells.*

While the death rates for many types of cancer are declining (1), likely due to the emphasis on lifestyle changes for the prevention of cancer, as well as better screening and earlier detection, death rates are still alarmingly high, particularly for certain types of cancers. Mortality rates are reported to be increasing for liver cancer and melanoma of the skin among men and for uterine cancer among women, while mortality rates for pancreatic cancer continue to rise among both men and women (1). Another important facet is the increasing incidence of obesity in the United States, which has been linked to cancer (1). For these reasons, the need for new chemotherapeutic agents is still at critical levels.

A current approach to cancer drug development is to target specific altered cell signaling pathways in tumor cells in contrast to classical chemotherapeutic agents that kill rapidly dividing cells and cause untoward side-effects. 4-phenyl-3-butenoic acid (PBA, Figure 1) is a small-molecule anti-inflammatory agent, which has been shown to be an irreversible inhibitor of peptidylglycine α-amidating monooxygenase (PAM) (2, 3). Additional studies have indicated that PBA could inhibit PAM activation in tumorigenic cell lines, decrease tumorigenic cell growth and reverse the transformed phenotype (4). The methyl ester derivative of PBA was effective at ~10-fold lower concentration (4). In light of this, we have identified specific cell signaling pathways that control cell growth and apoptosis that are modulated by PBA and PBA-Me. p38 MAPK and JNK are stress-activated members of the MAPK family that play important roles in the control of cell proliferation in a wide variety of cell types (5, 6). Altered expression or activation of these MAPKs has been observed in numerous tumorigenic cells and human cancers (6-12). An ideal chemotherapeutic agent would selectively modulate these altered pathways in tumor cells with substantially reduced effects on normal cells of the body, thereby leading to diminished toxicity. Previous work has shown that PBA and PBA-Me can selectively increase p38 MAPK activation in tumorigenic cells at concentrations that do not affect non-tumorigenic cells (13). Additionally, PBA can decrease activation of JNK in tumorigenic cells at concentrations that do not decrease activation in non-tumorigenic cells (13). PBA has also been shown to increase gap junction intercellular communication (GJIC) in tumorigenic cells (4,

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Key Words: PBA, vorinostat, p38 MAPK, JNK, gap junction.

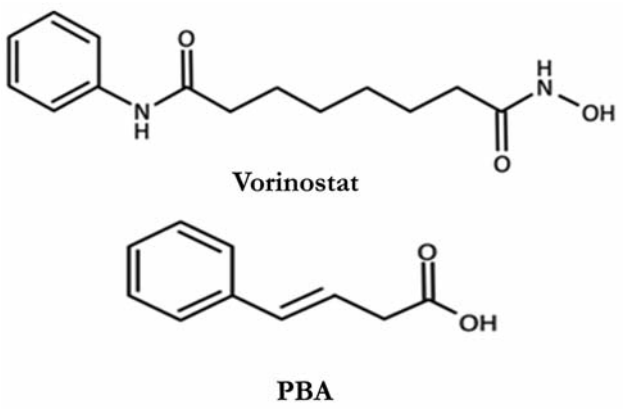

Figure 1. *Structure of vorinostat (suberoylanilide acid) and PBA (4-phenyl-3-butenoic acid, also referred to as trans-styrylacetic acid).*

13), which is of key importance as many tumorigenic cells lose the ability to communicate through gap junctions and proliferate without control. PBA also exhibits anti-inflammatory effects (14), which is of note, as there is a reported link between cancer and inflammation (15). Overall, these findings, along with the finding that PBA and PBA-Me can decrease cell growth in tumorigenic cells, indicate that PBA or its more potent methyl ester analog may have utility in treating cancer.

Vorinostat (Figure 1), also known as suberoylanilide acid (SAHA), is an anticancer agent in the drug class histone deacetylase (HDAC) inhibitors that shares structural features with PBA. Vorinostat is a class I, II and IV HDAC inhibitor. HDACs are epigenetic regulators responsible for the deacetylation of histones, as well as non-histone proteins (16). The *in vitro* effects of vorinostat therapy are well-characterized and include induction of mitotic cell death among drug-resistant neuroblastoma cells with non-functional p53 (17). Additionally, sensitization of neuroblastoma cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-induced apoptosis has been noted with vorinostat (18), as well as potentiation of the antineoplastic effect of radiation therapy, which occurs *via* down-regulation of a DNA repair enzyme (19). Vorinostat is marketed under the trade name Zolinza® and is indicated as 2nd- to the 3rd-line therapy for cutaneous manifestations of T-cell lymphoma (20).

The aim of the present study was to compare PBA with structurally-similar vorinostat in various *in vitro* tumor cell models. Comparisons were made with regards to effects on intracellular signaling, cell-cell communication and cell growth. To test selectivity of the two agents, non-tumorigenic cell models were also utilized. We show that PBA and vorinostat have similar effects on cell signaling and cell growth of tumorigenic cell lines. PBA-Me was effective in inhibition of cell growth at 10-fold lower concentration then PBA. Furthermore, while previous studies have shown that vorinostat can increase GJIC (21), we are the first to compare these effects with PBA in WBras1 cells. Both compounds showed selectivity for H2009 carcinoma cells *versus* normal HBE lung cells in terms of growth. Experiments using non-tumorigenic WB-neo_3 cells suggest selectivity of PBA and PBA-Me for tumorigenic *ras*-transformed cells *versus* WB-neo_3 cells in terms of growth and JNK inhibition, which may translate to a favorable adverse effect profile when administered systemically.

Materials and Methods

Cell culture. WBneo and WBras cells were derived from WB-F344 rat liver epithelial cells (22) *via* transfection of H-*ras* oncogene (WBras) or blank plasmid (WBneo) and were obtained from Dr. James Trosko at Michigan State University; these cells were subcloned from single cells to obtain the WBneo_3 and WBras_1 cell lines, and were used between passages 5 and 18. WBneo_3 and WBras_1 cells were grown in α-modification of Eagle's medium (α-MEM) supplemented with 2 mM L-glutamine and 5% FBS. G418 in PBS was added to the cell growth media at a concentration of 500 μM for cell culturing, but was omitted for experiments. H2009 human lung carcinoma cells were obtained from the American Type Culture Collection (Manassas, VA, USA) and were grown in Roswell Park Memorial Institute (RPMI) medium supplemented with 2 mM L-glutamine and 10% FBS, and used between passages 38 and 53. Human bronchial epithelial (HBE) cells obtained from Cell Applications, Inc. (San Diego, CA, USA) were grown in Cell Applications, Inc. bronchial/tracheal epithelial cell growth medium (San Diego, CA, USA) and used at passage 2. For experiments, confluent cells were subcultured by utilizing 0.25% trypsin and plated at approximately 15% confluence. Cells were incubated in an atmosphere of 5% CO_2 at 37˚C.

Cell growth. Cells were plated into individual 9.5 cm^2 round plates at 10^5 cells per dish. After 24 h, cells were subjected to treatment with PBA, vorinostat or vehicle control (n=4 for each treatment) on day 1 (0 hours). After 24 h, cells were treated again with PBA or vehicle control. Cells in the vorinostat treatment group(s) did not receive a second treatment as doing so results in insufficient cell density to perform the assay (unpublished observations). Per the package insert for vorinostat, the pharmacologically relevant concentration of vorinostat is 2,000 nM, with the drug being approximately 71% plasma protein bound, which gives a free drug concentration of approximately 500 nM (20). Above this, unacceptable toxicity occurs. Preliminary studies revealed that single treatments for 48 h at 500 nM were able to produce robust effects in various assays, while concentrations above this caused a marked decrease in cell density that precludes the performance of various assays (unpublished observations). Similar decreases in cell density were also seen in cells treated with vorinostat twice. Therefore, we found 500 nM to be the optimum dose for vorinostat, which can produce robust effects and still allow for significant cell density for performance of our assays. Likewise, the maximum non-toxic dose for PBA is 617 μM as previously reported (4); this dose given twice as indicated above provides for robust effects while minimizing toxicities. At 48 h post-treatment, cells were suspended by incubation with trypsin and counted using the BioRad® TC-10™

cell counter (Bio-Rad Laboratories, Hercules, CA, USA). Alternatively, growth was measured by a Neutral Red viability assay (4). Cells grown in 48-well culture plates were treated with 61.7 μM of PBA-Me dissolved in DMSO for 48 h and incubated for 3 h at 37˚C with 0.15% Neutral Red dye added to the culture medium. Cells were washed of external dye once with 1 mM Ca^{2+}/1mM Mg^{2+} PBS and twice with PBS, followed by addition of 1 ml of extraction solution comprised of 50% ethanol and 1% acetic acid. Absorbances of samples (n=6) were read at 540 nm on a Tecan plate reader (Phenix Research Products, Hayward, CA, USA).

Gap junction intercellular communication assay. A fluorescent dye transfer assay was utilized to assess gap junction intercellular communication (GJIC) as previously described (13). Cells were plated in 9.5 cm^2 cell culture dishes at a density that allowed equal cell density for each treatment group upon performance of the GJIC assay. Treatment was carried out in duplicate for 48 h with PBA, vorinostat or vehicle control as previously described. Additionally, one group of cells was treated in duplicate with dieldrin one hour before commencement of the assay. A fluorescent dye transfer assay was utilized to quantify GJIC (23). In brief, cells were washed with Ca^{2+}/Mg^{2+} PBS (0.25 mM each) followed by PBS, followed by the addition of lucifer yellow dilithium salt purchased from Sigma (L0259; St. Louis, MO, USA). Approximately 8 to 11 cuts were then made in each dish with a scalpel, followed by incubation for 90 sec at room temperature to allow for diffusion of the dye into compromised cell membranes. Cells were then washed with PBS and then Ca^{2+}/Mg^{2+} PBS (1 mM each) and fixed with 4% paraformaldehyde for approximately five hours. Pictures were then taken of cut areas of equal cell density in white light viewing mode and fluorescent viewing mode. These pictures were then randomized, with fluorescent cells being counted by an external observer. Counts were also obtained from cells treated with the GJIC inhibitor dieldrin; cells that were fluorescing in this group would only be fluorescing due to a compromised cell membrane. An average of these counts was then subtracted from all groups to obtain an accurate count of those cells exhibiting GJIC.

Cell signaling and western blotting. Cells were grown in 25 cm^2 flasks to roughly 40% to 50% confluence and subsequently treated in duplicate with drug or vehicle control (deionized water for PBA and DMSO for vorinostat) for 48 h as previously described. Cells were extracted by washing with PBS and incubating with a 2% SDS extraction buffer containing protease inhibitor cocktail (Sigma® P-8340) and 10 mM phenylmethylsulfonyl fluoride (PMSF) for 90 sec. Following this, cells were scraped utilizing a cell scraper. Extracts were then sonicated for thirty seconds. The lysate was then frozen in liquid N_2 and stored at –20˚C. Protein in each sample was quantified utilizing a DC Bio-Rad protein assay (Hercules, CA, USA). Equal amounts of protein for each sample along with pre-stained molecular mass markers were loaded onto 12% polyacrylamide gels, with electrophoresis being performed at 60 mV for approximately 2.5 h. Following electrophoresis, proteins were transferred to polyvinylidene fluoride (PVDF) membranes using either a wet transfer process or Biorad Turboblot™ transfer apparatus. Ponceau staining of PVDF membranes was utilized to confirm equal protein loading for each lane. Membranes were blocked using 4% non-fat dry milk-based blocking buffer and incubated with (primary) antibodies, which contained specificity to phosphorylated or non-phosphorylated sites where applicable, followed by incubation with alkaline phosphatase-linked (AP) secondary antibodies. Rabbit polyclonal antibodies to p38 MAPK, phospho-p38 MAPK (Thr180/Tyr182), JNK, phospho-JNK (Thr183/Tyr185), phospho-HSP27 (Ser82) and alkaline phosphatase (AP) conjugated anti-rabbit IgG were utilized, all of which were purchased from Cell Signaling Technology (Danvers, MA, USA). Blots were developed by either using a Bio-Rad AP conjugate development kit or Lumi-Phos™ WB development followed by exposure to Kodak™ BioMax® Light Film. Un-Scan-it densitometric software was utilized to quantify changes in band density (Silk Scientific, Orem, UT, USA)

Statistics. All statistical analyses were performed using the Statistix® software by Analytical Software (Tallahassee, FL, USA). Analysis of variance (ANOVA) with subsequent Tukey *post-hoc* test was performed on all data with a $p<0.05$ considered statistically significant.

Results

PBA and vorinostat inhibit cell growth in tumorigenic cell lines. We first examined the ability of these two agents to decrease cell growth in tumorigenic cell lines. As previously described, treatment was carried out with PBA and vorinostat at 617 μM and 500 nM, respectively. Treatment of tumorigenic WB*ras*$_1$ cells with PBA or vorinostat at these non-toxic doses for 48 h resulted in a marked decrease in cell growth compared to vehicle-treated controls (Figure 2a). This decrease in cell growth is more dramatic secondary to vorinostat treatment than PBA treatment and is significantly different as confirmed by the Tukey test ($p<0.05$). Likewise, treatment of H2009 human lung carcinoma cells with PBA or vorinostat at these doses for 48 hours also resulted in a marked decrease in cell growth (22% decreased for PBA, 26% decreased for vorinostat, $p<0.01$ (Figure 2c). We next monitored the effects of PBA and vorinostat on cell growth in non-tumorigenic cells to assess their potential selectivity for tumorigenic cell types. We used WBneo3 cells for this purpose as they are the non-tumorigenic analog of WBras1 cells and normal human bronchial epithelial (HBE) cells to compare with the H2009 cells. Treatment of non-tumorigenic HBE cells with PBA or vorinostat showed no significant differences in cell growth at 48 h compared to vehicle controls (Figure 2d). Interestingly, vorinostat at concentrations of both 250 and 500 nM caused a decrease in the growth of non-tumorigenic WB*neo*$_3$ cells at 48 h ($p<0.05$ compared to control), while PBA at 617 μM did not (Figure 2b). PBA-Me at a 10-fold lower concentration than PBA significantly decreased cell growth of WBras1 (Figure 2e) but not WB*neo*$_3$ at 48 h (Figure 2f), as previously demonstrated for 4 days of treatment (4).

PBA and vorinostat have similar effects on growth signaling pathways in tumorigenic cell lines. Previous studies have shown that vorinostat and PBA have the

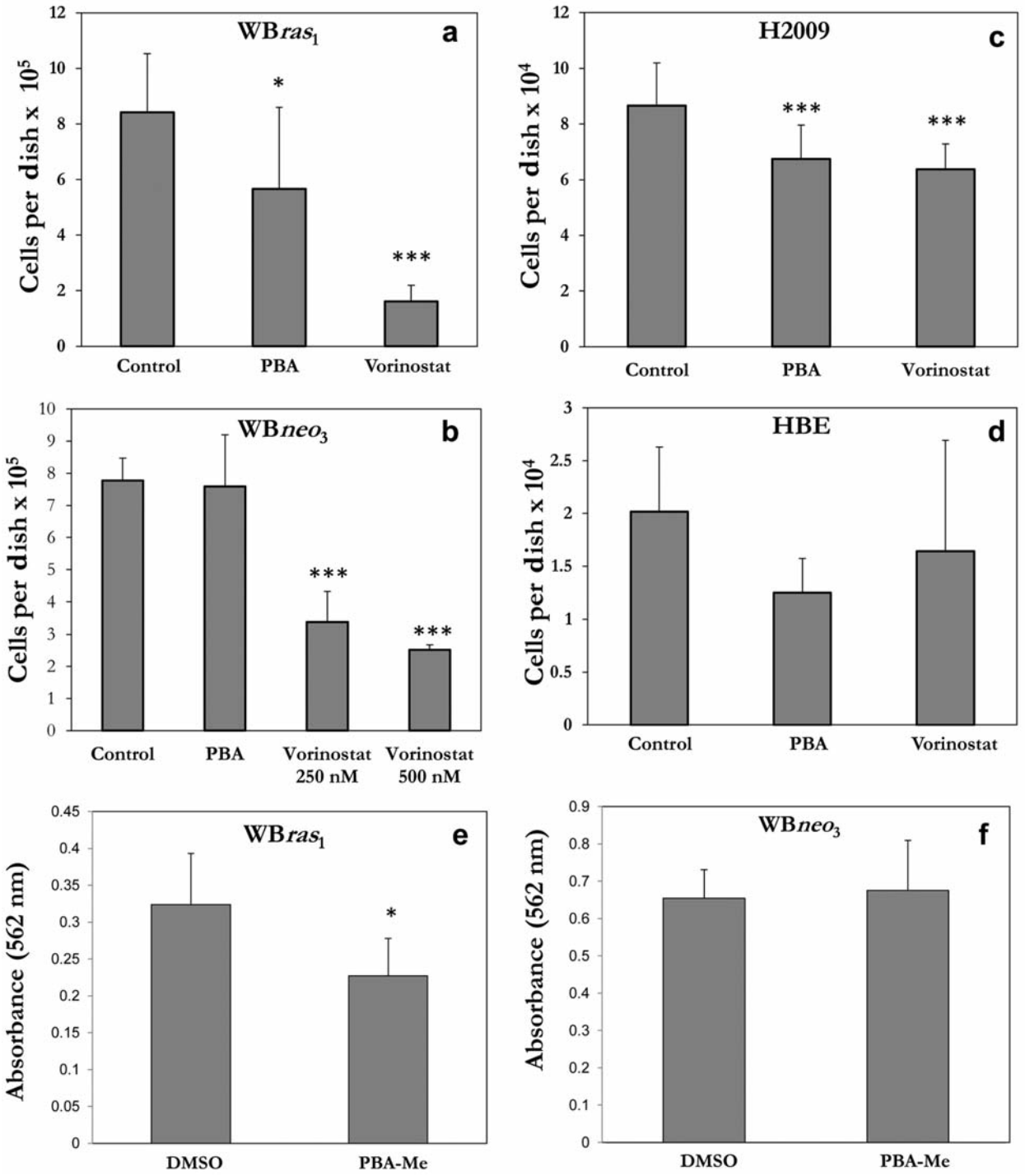

Figure 2. *(a) Cell growth of WBras1 cells treated for 48 h with PBA (617 μM), vorinostat (500 nM) or vehicle control (water + DMSO). Cells were initially plated at a density of 2.7×10^5 cells per dish. Treatment was performed 24 h following plating and a second treatment (PBA only) was performed 48 h after plating. Cell counting was performed 48 h after the initial treatment. (b) Cell growth of WBneo3 cells treated for 48 h with PBA (617 μM) or vorinostat (250 or 500 nM). Cells were plated and treated as described in (a). *p<0.05, ***p<0.01, compared to control. (c) Cell growth of H2009 cells treated for 48 h with PBA (617 μM), vorinostat (500 nM) or vehicle control (water + DMSO). Cells were plated and treated as described in (a). ***p<0.01, compared to control. (d) Cell growth of HBE cells treated for 48 h with PBA (617 μM) or vorinostat (500 nM). Cells were plated and treated as described in (a). Significant differences were not found. (e) Cell growth of WBras1 cells treated for 48 h with PBA-Me (61.7 μM) or vehicle control (DMSO). Treatment was performed 24 h following plating. Cell counting or the Neutral Red assay described in Methods were performed 48 hours after treatment. Error bars represent the standard deviation within experiment replicates.*

ability to increase phosphorylation of p38 MAPK in various tumor cell models (13, 24-27). In the present study we compared the cell signaling effects of PBA and vorinostat under similar conditions in WBras1 and H2009 human carcinoma cells. Treatment was carried out for 48 h with both agents (Figure 3a). PBA and vorinostat both increased phosphorylation of p38 MAPK with no significant difference between PBA and vorinostat at 617 μM and 500 nM, respectively (Figure 3a and 3b). Downstream of p38 MAPK, increased phosphorylation of HSP27 was seen with both treatments with PBA causing a greater than 6-fold increase in phosphorylation and vorinostat causing an approximately 3-fold increase in phosphorylation (Figure 3c). Results obtained *via* treatment of vorinostat or PBA on JNK showed that both agents significantly decreased JNK phosphorylation in WBras1 tumorigenic cells (Figure 4), as was previously demonstrated for PBA and vorinostat (13, 25).

Vorinostat, but not PBA, decreases JNK phosphorylation in non-tumorigenic cell lines. Following the confirmation that PBA and vorinostat have similar effects on cell signaling in tumorigenic cells, it was desired to monitor their effects on cell signaling in the non-tumorigenic WB*neo*$_3$ cells, to assess their potential selectivity for tumorigenic cell types. This was accomplished by carrying-out experiments in WBneo3 cells in the same manner as WBras1 cells, wherein cells received two treatments of PBA (617 μM) and one treatment of vorinostat (500 nM) for 48 h. Treatment with PBA twice for 48 h did not cause a significant change (p=0.2416) in phosphorylation of the stress activated kinase JNK in WB*neo*$_3$ cells (Figure 5). Treatment with vorinostat at 500 nM for 48 h, however, significantly decreased JNK phosphorylation in WB*neo*$_3$ cells (Figure 5). At these same concentrations and time points, treatment with either PBA or vorinostat led to decreased phosphorylation in JNK in tumorigenic cell lines, as described above. Changes in p38 MAPK phosphorylation and HSP27 phosphorylation were not seen when WB*neo*$_3$ cells were treated with PBA or vorinostat at the same concentrations used for WBras1 and H2009 cells (data not shown).

Vorinostat and PBA both increase GJIC in tumorigenic cells. A fluorescent dye transfer assay was used to quantify the number of cells that were expressing functional gap junctions following treatment of WBras1 cells for 48 h with PBA or vorinostat. Both PBA and vorinostat caused an increase in GJIC compared to control (Figure 6). Per unit area, it was found that an average of 15.7 vehicle treated cells produced fluorescence compared to 53.2 and 49.2 for PBA and vorinostat, respectively. Overall, this translates to a ~3.4-fold increase in GJIC with PBA treatment and ~3.1-fold increase in GJIC with vorinostat treatment.

Discussion

The results presented here indicate that while cell signaling, cell growth and GJIC effects of PBA and vorinostat are similar, there are some noteworthy differences. While the relative potencies of the two agents are different, the dose of PBA used in the present study is non-cytotoxic, as previously reported (4). In addition, the methyl ester of PBA is effective at growth inhibition (Figure 2e-f) and p38 MAPK modulation (4, 13) at 10-fold lower concentration than PBA, likely due to its ability to penetrate into cells more effectively. Despite the higher doses of PBA and PBA-Me, they none-the-less show selectivity for tumorigenic *versus* non-tumorigenic cells with respect to growth, as well as for JNK signaling in the case of PBA, making them good candidates for *in vivo* studies in animal models of tumorigenesis. In addition, we recently reported effects of an even more potent acrylate compound, AOPHA-Me, that inhibits growth and modulates JNK and p38 MAPK in RAW 264.7 cells at 50- to 100-fold lower concentration than PBA (28).

In H2009 cells, the effects of PBA and vorinostat on cell growth are similar and not significantly different at the time point of 48 h (Figure 2b). Both drugs did not significantly inhibit the normal HBE lung cells under similar conditions (Figure 2d) indicating that they are similarly selective for inhibiting the H2009 carcinoma cells compared to non-tumorigenic lung cells. At the time point of 48 h vorinostat does, however, have a more robust effect on cell growth than PBA and PBA-Me in WBras1 cells at the concentrations used. Our previous studies using WBras1 cells showed that a single dose of both PBA and PBA-Me have a more pronounced effect on growth compared to vehicle controls at longer times points following treatment with PBA, >60% inhibition at 4 days (4), at the same concentrations used in the present study. This previous study also showed that the difference in effectiveness of PBA and PBA-Me on tumorigenic WBras1 cells compared to non-tumorigenic WBneo3 cells was also more dramatic following 4 days of treatment (4). A treatment time of 48 h was kept constant in the present study in order to more readily make comparisons with vorinostat on growth, GJIC and p38 MAPK and JNK signaling. At the concentrations of drugs used in the present study to inhibit growth of WBras1 cells, vorinostat had a pronounced effect on WBneo3 cell growth, whereas PBA (Figure 2a and b) and PBA-Me (Figure 2e and f) did not. While higher concentrations of PBA would be predicted to inhibit WB*neo*$_3$ cell growth based on our previously published study using a triple dose of PBA (4), taken together, our studies highlight a potential concentration 'therapeutic window' for PBA and PBA-Me, to achieve selectivity for tumorigenic cells *versus* non-tumorigenic cells that may limit adverse effects *in vivo*.

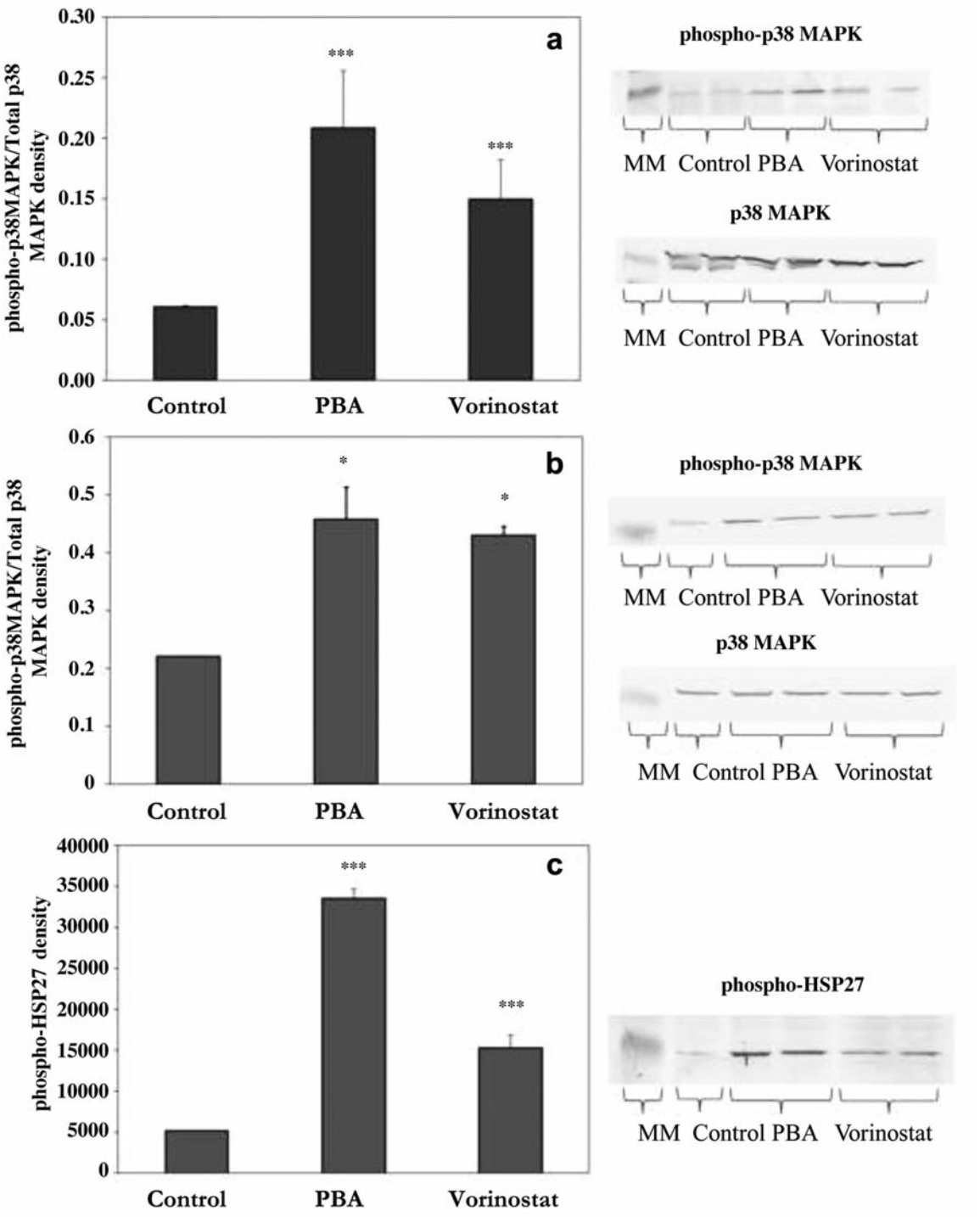

Figure 3. *(a) Both PBA and vorinostat increased p38 MAPK phosphorylation at the Thr180/Tyr182 activation site compared to control. H2009 cells were treated for 48 h (prior to extraction) with PBA (617 μM), vorinostat (500 nM) or vehicle control (water + DMSO). Phosphorylated p38 MAPK levels were normalized to total p38 MAPK levels. Western blotting was performed with equal protein loading confirmed via Ponceau staining. Western blots were developed using alkaline phosphatase-linked secondary antibodies, blots were scanned into a computer and analyzed using densitometry software. (b) Both PBA (617 μM) and vorinostat (500 nM) increased p38 MAPK phosphorylation compared to control (water + DMSO) in WBras1 cells that were treated for 48 h. Phosphorylated p38 MAPK levels were normalized to total p38 MAPK levels. Western blotting and data analysis was similar to (a). (c) PBA (617 μM) and vorinostat (500 nM) both increased phosphorylation of HSP27 at the Ser82 site compared to control (water + DMSO) in WBras1 cells that were treated for 48 h. Western blotting and data analysis was similar to (a) and (b). *p<0.05, ***p<0.01, compared to control. Error bars represent the standard deviation within experiment replicates.*

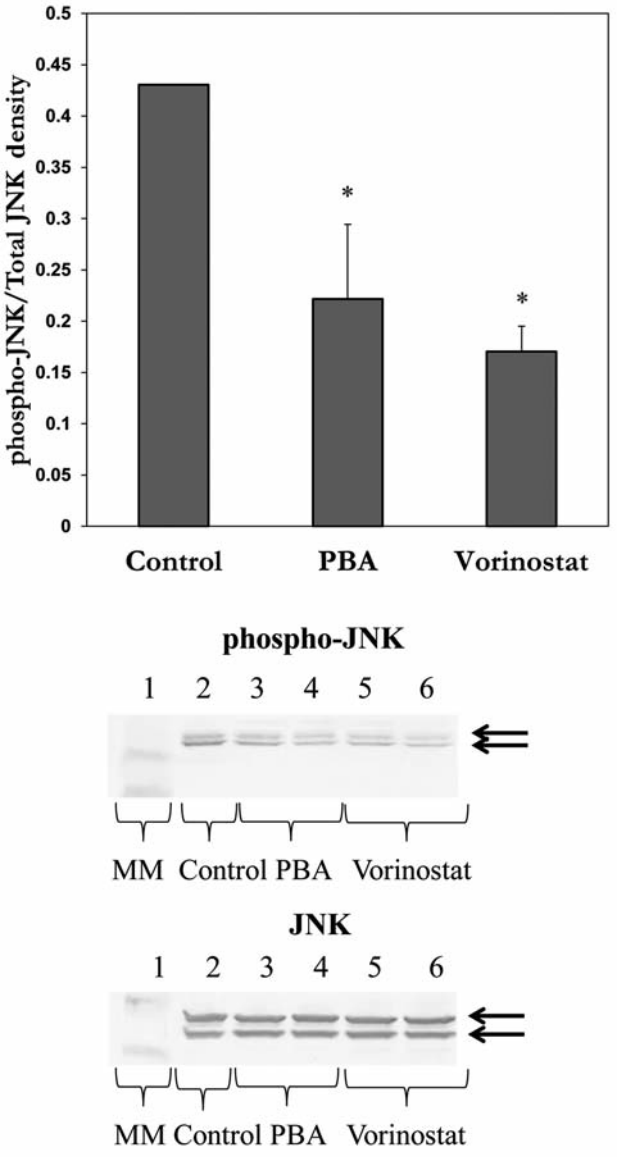

Figure 4. *Both PBA and vorinostat decreased JNK phosphorylation at the Thr183/Tyr185 activation site compared to control. WBras1 cells were treated for 48 h prior to extraction with PBA 617 μM, vorinostat 500 nM or vehicle control (water + DMSO). Blots were scanned and analyzed with densitometric software, with phospho-JNK levels being normalized to total JNK levels, * p<0.05, compared to control. Error bars represent the standard deviation within experiment replicates.*

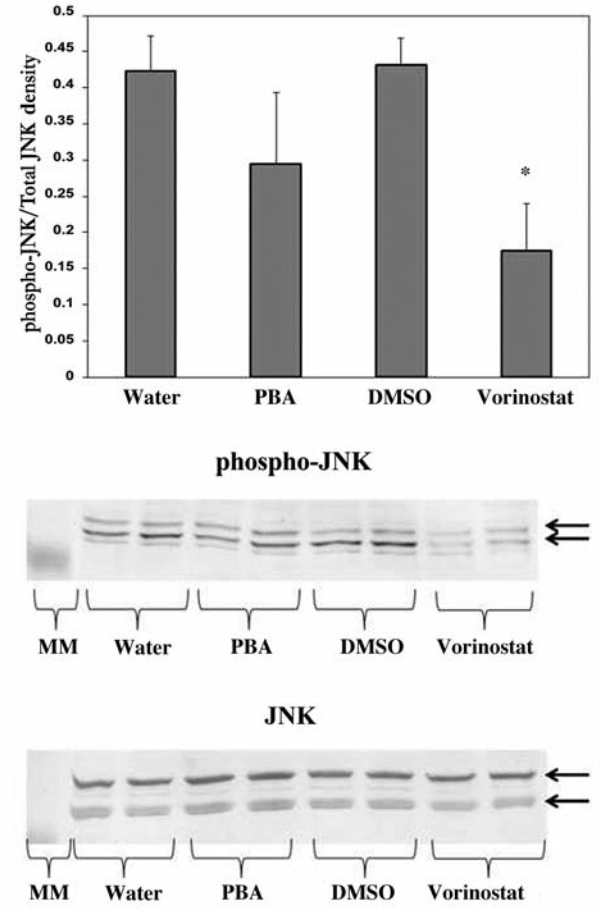

Figure 5. *Vorinostat, but not PBA, decreased JNK phosphorylation at the Thr183/Tyr185 activation site in non-tumorigenic WBneo3 cells. Cells were treated with PBA (617 μM), vorinostat (500 nM), water (vehicle control for PBA), or DMSO (vehicle control for vorinostat) for 48 h. Vorinostat caused a 60% decrease in JNK activation compared to DMSO. *p<0.05, compared to control. Error bars represent the standard deviation within experiment replicates.*

It has been shown previously by Bali *et al*. that vorinostat can induce apoptosis in breast cancer cells at doses of 1,000, 2,000 and 5,000 nM (29). This specific study showed an approximately 10-15% increase in apoptosis confirmed *via* Annexin V levels at 1,000 nM with dose dependent increases seen up to approximately 45% with 5,000 nM treatment (29). While the doses in the Bali *et al*. study are higher than those reported in the current study, it should be noted that different cell lines were used (SKBR-3 and BT-474 breast cancer cell lines) (29). In a model of non-small-cell lung carcinoma (NSCLC), it has been shown that vorinostat can induce apoptosis in A549 and NCI-H460 cells at doses greater than 1,000 nM but not 500 nM (30). Using a TdT-FragEL DNA fragmentation detection kit, we reported that PBA at 617 μM causes an approximately two-fold increase in apoptosis (13). This increase in apoptosis is modest (approximately 8%) and is similar to the aforementioned study, wherein vorinostat caused a 10-15% increase in apoptosis in breast cancer cells (29). This points to the inhibition of cell growth following PBA and vorinostat treatment reported herein as not being solely from

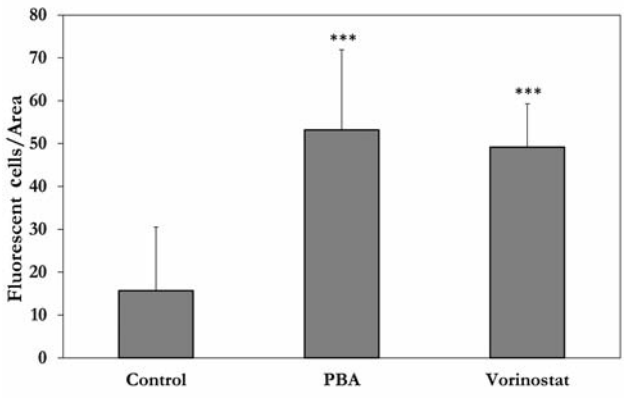

Figure 6. *Both PBA and vorinostat increased GJIC compared to vehicle controls. WBras1 cells were treated for 48 h prior to performance of the GJIC assay with PBA (617 μM), vorinostat (500 nM) or vehicle control (water + DMSO). Fluorescent dye transfer was utilized to measure functional gap junctions. ***p<0.01 compared to control. Error bars represent the standard deviation within experiment replicates.*

the induction of apoptosis. Previous reports also have shown that at 5 days post-treatment, PBA causes a more robust decrease in cell growth than at 48 h (13, 31).

Recent clinical studies are bringing to light the toxicities associated with vorinostat therapy. Vorinostat, while once promising (32, 33), has been somewhat of a disappointment with regards to its effectiveness and tolerability. Some examples of this include a clinical trial of vorinostat in combination with other established chemotherapy agents in the treatment of advanced NSCLC, which was terminated early (34). Another example was a phase IIa study with vorinostat-alone in the treatment of lower-risk myelodysplastic syndromes (MDS). This study was terminated due to lack of efficacy and increased adverse events (35). In a phase I/II study of vorinostat in combination with erlotinib in the treatment of relapsed/refractory NSCLC, the study was terminated due to "a lack of substantive efficacy, slow accrual and overall tolerance in patients treated to date" (36). A study investigating vorinostat and trastuzumab in treating patients with metastatic or locally recurrent breast cancer was terminated early due to a low response rate in a pre-planned efficacy evaluation (37). Vorinostat was not effective in combination with decitabine in a phase I study in patients with acute myeloid leukemia (AML) or MDS (38). Also of concern is that vorinostat was found to increase a subjects' cardiologic QTc interval in a trial in which patients had advanced forms of cancer (39). Therefore, vorinostat has shown limited clinical utility in the treatment of cancer, particularly solid tumors, while exhibiting adverse effects to an extent that are intolerable to a number of cancer patients.

Vorinostat works through HDAC classes I, II and IV. Our preliminary studies show that PBA has HDAC inhibitory activity for classes I and II (unpublished observations, manuscript in preparation). A preliminary comparison of the two agents has been performed in which it was shown that both agents showed similar acetylation patterns on histones H2B, H3 and H4 (40). The results of the current study indicate that PBA shares some similarities with vorinostat. Differences in cell signaling and cell growth of non-tumorigenic cells may be indicative of PBA working through different HDAC isoforms than vorinostat. Both agents also cause a decrease in phosphorylation of p53 at ser15 (unpublished data) indicating that they are able to increase phosphorylation of p38 MAPK in a wild-type p53-induced phosphatase 1 (WIP-1) independent manner. Further studies are needed to fully characterize the p38 MAPK activation caused by PBA and vorinostat.

The current study showed that PBA and vorinostat have overall similar effects on cell signaling, cell communication and cell growth in tumorigenic cells. However, at the concentrations used in this study, which were effective in *ras*-transformed cells, vorinostat inhibits cell growth in the WB-*neo*$_3$ non-tumorigenic cells in contrast to PBA and PBA -Me. Vorinostat also affected JNK signaling in WB-*neo*$_3$ cells, whereas PBA did not. While further studies are required at additional concentrations and time points, these results suggest that PBA and PBA-Me may be more selective for tumorigenic *versus* non-tumorigenic cells within a given therapeutic range. Therefore, *in vivo* studies are needed to assess whether PBA may be a suitable alternative to those patients unable to tolerate vorinostat therapy. The effects shown on GJIC were expected as other HDAC inhibitors, such as valproic acid, have recently been shown to increase GJIC in tumorigenic cells (41). This lends these agents additional utility when used as an adjunct to a prodrug due to the bystander effect (41). For these reasons, the development of PBA and/or its more potent analogs PBA-Me, or AOPHA-Me as anticancer agents, may be warranted as a potentially favorable option or as an alternative agent if cancer cells develop resistance to vorinostat.

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